IN VITRO PROSTAGLANDIN E2 SYNTHESIS IN THE SALIVARY GLANDS OF THE FEMALE LONE STAR TICK, *Amblyomma americanum* (L.)

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Table Page

LIST OF ABBREVIATIONS

AA	arachidonic acid
AC	adenylate cyclase
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CAT	chloramphenicol acetyl transferase
cDNA	complementary deoxy ribonucleic acid
CHAPS	3-[(3-cholamidopropyl)dimethylammonio-1-propane sulfate]
CoA	coenzyme A
COX	cyclooxygenase
$cPLA_2$	cytosolic phospholipase A_2
DAT	diacylglycerol acyltransferase
DNA	deoxyribonulcleic acid
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
ETYA	(5, 8, 11, 14-eicosatetraynoic acid)
GC/MS	gas chromatography/mass spectrometry
HETE	hydroeicosatetraenoic acid
HPETE	hydroperoxyeicosatetraenoic acid

IP_3	inositol 3 phosphate
IL1β	interleukin 1 β
$iPLA_2$	calcium-independent phospholipase A_2
Kb	kilobase
KDa	kilodalton
LAT	lysophosphatidyl acyltransferase
LPC	lysophosphatidylcholine
M	molar
MAPK	mitogen-activated protein kinase
MBD	membrane binding protein
μCi	microcurie
μM	micromolar
mM	millimolar
MOPS	morpholinopropane sulfonic acid
NSAID	nonsteroidal anti-inflammatory drugs
O.D.	optical density
PC	phosphatidylcholine
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PG	prostaglandins

PGA_2/B_2	prostaglandin A_2/B_2
PGD_2	prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGHS	prostaglandin H synthase
PGI_2	prostacyclin
PI	phosphatidylinositol
PKC	protein kinase C
PL	phospholipids
PLA_2	phospholipase A_2
PLC	phospholipase C
R_f	Relative mobility
RIA	radioimmunoassay
RNA	ribonuleic acid
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SEM	standard error of the mean
$sPLA_2$	secretory phospholipase A_2

TG	triglycerides
TLC	thin layer chromatography

CHAPTER I

INTRODUCTION

I-I Eicosanoids Metabolism in Mammalian Systems

I-I-I Eicosanoids Nomenclature and Biological Importance

The name eicosanoid refers to unsaturated lipids, having 20 carbons, derived mainly from eicosatetraenoic acid, arachidonic acid (AA) (C20: 4), or similar polyunsaturated fatty acids such as 20:3 and 20:5 (Granstrom & Kumlin, 1987).

Arachidonic acid exists in human plasma at a concentration of about 10 μM mostly bound to albumin and other proteins (Liu & Weller, 1990). After entering cells, arachidonic acid is mainly esterified into membranous phospholipids and neutral lipids. Only a small portion of the AA remains in a free form inside the cell (Liu & Weller, 1990).

Upon release from membranes, free AA can be metabolized by oxygenation via three major enzymatic pathways depending on the cell type. The first is the prostaglandin synthase or the cyclooxygenase pathway, which forms prostaglandins (PGs), and thromboxanes (Granstrom & Kumlin, 1987). The second is the lipoxygenase pathway that generates leukotreines and hydroperoxide derivatives of AA called hydroperoxyeicosatetraenoic acids or HPETEs that are rapidly converted to hydroxyeicosatetraenoic acid or HETEs. The third route is the cytochrome P_{450} epoxygenase pathway that yields hydroxy- and epoxy-derivatives of AA (Needlman *et al.*, 1986).

A schematic diagram of the three major pathways of AA metabolism in cells and the release and the reincorporation of AA into the membranes is shown in Figure 1.1.

Eicosanoids may act as both intracellular second messengers and local mediators utilized by the same tissues that synthesize them, autacoids, or by the surrounding tissues (Samuelsson *et al.*, 1978; Piomelli, 1993). Eicosanoids are distinguishable from stable circulating peptide factors by being released shortly after biosynthesis and lack of storage. Moreover, eicosanoids do not exert wide-ranging systematic effects due to their rapid degradation into inactive metabolites in the circulation or in tissues (Liu & Weller, 1990).

In addition to their short half-life, and because of their very high potencies, eicosanoids typically occur only in minute amounts in most body tissues and fluids. The endogenous concentration of prostaglandins in peripheral plasma in human remains less than 2 pg/ml (Granstrom & Kumlin, 1987).

Eicosanoids exhibit a wide range of biological effects within cells determined by the type and the amount of the effector in each cell type. Prostaglandin E_2 (PGE_2) and prostaglandin D_2 (PGD_2) are potent inhibitors of platelet aggregation. PGE_2 has immunosuppressive and anti-inflammatory effects including the inhibition of granulocyte, monocyte, and macrophage functions, inhibition of T lymphocyte activation and lymphokine production, and induction of lymphocyte unresponsiveness (Goodwin, 1985; Lewis, 1983). In addition, PGE_2 potently induces apoptosis in T and B cells (Mastino *et al.*, 1992). Both PGE_2 and prostacyclin PGI_2 are potent vasodilators (Goodwin, 1985). HETEs and leukotrienes can mediate granulocyte chemotaxis, degranulation, and other functions (Bray *et al.*, 1981). Cytochrome P_{450} metabolites of

Fig.1.1 the cellular main pathways of AA release and metabolism, from (Piomelli, 1993)

a) Hydrolysis of PL and AA release via PLA_2

b) PLC activated release of AA

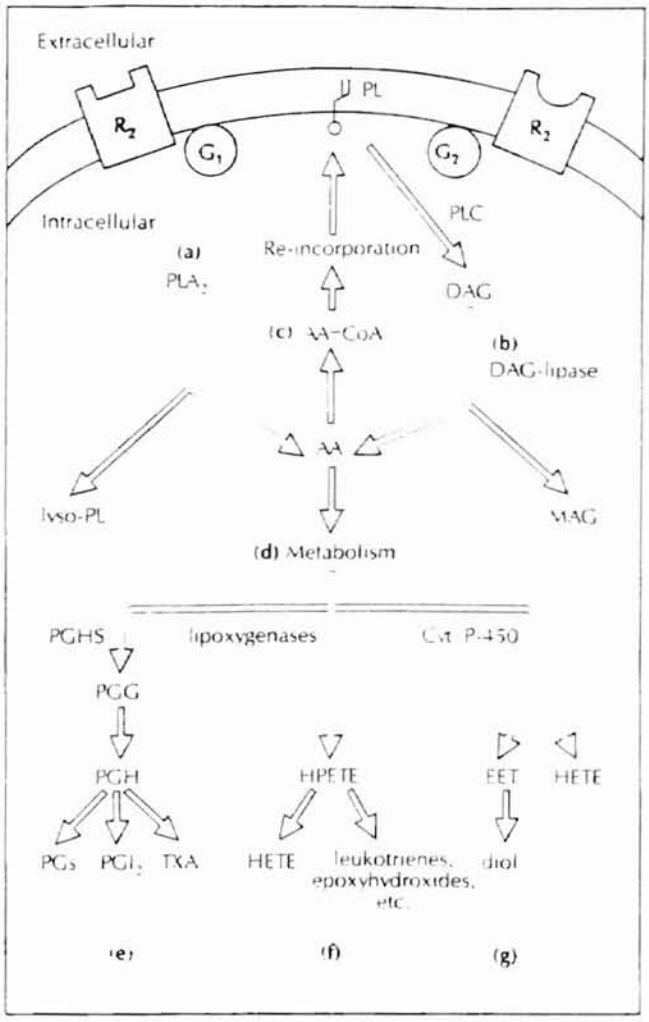
c) Reincorporation of AA into PL

d) Metabolism of AA via

e) PGHS

f) Lipoxygenase

g) Cytochrome oxidase P_{450}


Extracellular
PL
R2
G1
G2
R2
Intracellular
PLC
(a)
PLA2
Re-incorporation
DAG
(c) AA-CoA
(b)
DAG-lipase
AA
lyso-PL
MAG
(d) Metabolism
PGHS
lipoxygenases
Cyt P-450
PGG
PGH
HPETE
EET
HETE
PGs PGI2 TXA
HETE
leukotrienes, epoxyhydroxides, etc.
diol
(e)
(f)
(g)

AA inhibit platelet aggregation and promote vasodilation (Murphy *et al.*, 1988).

I-I-II Prostaglandin H Synthases PGHS [EC.1.14.99.1]

PGHS, or cyclooxygenase COX, was identified over twenty years ago and shown to mediate the rate-limiting step in prostaglandin PG biosynthesis from arachidonic acid (AA). In the early 1990s, another isoform of this enzyme was discovered. The two isoforms are named PGHS-1 and PGHS-2, or COX-1 and COX-2, with respect to the time order of their identification (Dubois *et al.*, 1998).

It was shown that PGHS-1 is constituitively expressed and is principally responsible for the biosynthesis of PGs involved in homeostatic regulation. On the other hand, the expression of PGHS-2 in many cells is inducible and is primarily involved in producing PG in response to inflammatory cytokines, growth factors, v-src and tumor promoters (Bauer *et al.*, 1997; Spencer *et al.*, 1999; Vane & Botting, 1997). Unlike PGHS-1, the PGHS-2 level is almost nondetectable in most cell types under resting conditions but can be rapidly induced in response to cytokines and endotoxin in macrophages, and to injury and interleukin in fibroblasts and endothelial cells (Chulada *et al.* 1996). Moreover, there is evidence that PGHS-1 and –2 function as separate biosynthetic systems. Both respond to different stimuli, utilize separate AA pools, and produce PGs with different functions (Chulada *et al.* 1996).

The cellular localization of the two enzymes is also different. Whereas PGHS-1 is mostly restricted to the luminal surface of the endoplasmic reticulum (ER) membrane with little or no activity localized in the outer and inner membranes of the nuclear envelope, PGHS-2 is concentrated and more active in the nuclear envelope as compared

to PGHS-1 (Chulada *et al.* 1996). The localization of PGHS-2 in the nuclear envelope possibly relates to its contact with a complex regulatory system leading to the expression of new proteins in response to cell environmental stimuli.

Apparently, distinct pools of AA exist. These pools are specifically available for either PGHS isoform but not both, thus separating the two pathways (Herschman, 1996; Chulada *et al.* 1996). Preference is observed in the utilization of exogenous vs. endogenous AA by PGHS-1 and –2, respectively (Dubois *et al.*, 1998). Using exogenous (AA) Chulada *et al.* (1996) showed that the activity of PGHS-1 was approximately 3-5 fold higher than PGHS-2 in mouse embryonic fibroblasts expressing both isoforms. Conversely, when endogenous AA was the only source of the substrate the activity of PGHS-2 was 5-fold higher than that of PGHS-1, although both isoforms showed lower activities compared to the usage of exogenous substrate.

PGHS isoforms share ~60% of primary amino acid sequence identity (Spencer *et al.*, 1999), and the x-ray crystal structures of the two proteins are virtually superimposable (Spencer *et al.*, 1999; Chulada *et al.* 1996). Even though PGHS-1 and PGHS-2 lack transmembrane domains, they are regarded as integral membrane proteins due to their solubilization by detergents but not chaotropic salts (Spencer *et al.*, 1999). The three dimensional structure of PGHS-1 revealed a homodimer where each monomer is comprised of three independent folding units: an epidermal growth factor-like (EGF-like) domain at the N-terminus of the protein, a membrane binding domain (MBD) and the catalytic domain (Picot *et al.*, 1994; Bazan *et al.*, 1996). The EGF-like modules constitute a significant portion of the dimer interface implying that the role of this domain is probably to aid in forming a stable dimer (Bazan *et al.*, 1996). The MBD is made up of

four amphipathic helices pointing outward along one side of the protein and thus forming a large hydrophobic patch on the surface of the protein. In the dimer, the MBD from each monomer is on the same side of the molecule. Together they form a large surface covered with hydrophobic amino acid side chains that anchor the enzyme to the membrane. The MBD penetrates only one leaflet of the membrane bilayer. Hence, PGHS-1 is a monotopic membrane protein (Spencer *et al.*, 1999). Three of the four helices of the MBD form the entrance to the hydrophobic channel of PGHS-1 that allows AA to gain access to the active site from the interior of the bilayer without passing through a polar environment. The third and the largest structural domain in PGHS-1 is the catalytic domain that assumes a canonical haem-dependent peroxidase fold similar to mammalian myeloperoxidases and to some peroxidases from lower eukaryotes (Vane & Botting, 1997).

The structure of PGHS-2 closely resembles that of PGHS-1 except that the former active site is slightly larger and can accommodate bigger structures than those for PGHS-1. Both PGHS isoforms are glycoproteins with a haem prosthetic group and molecular weight of about 70 KDa. The catalytic domains of PGHS-1 and –2 share 70% of the primary structure, whereas the MBDs are only 38% identical (Spencer *et al.*, 1999).

The two isozymes possess cyclooxygenase and peroxidase activities that are functionally separable, but linked because the oxidizing equivalents generated by the peroxidase reaction may be required to initiate cyclooxygenase turnover. The cyclooxygenase component of PGHS converts AA to hydroxyendoperoxide (PGG_2), and the peroxidase component reduces the endoperoxide to the corresponding alcohol (PGH_2), the precursor of all prostaglandins (Hsuanyu & Dunford, 1992). Although a

channel does not connect the active sites of cyclooxygenase and peroxidase, PGG_2, the product of the cyclooxygenation of AA is the substrate for peroxidase reduction to PGH_2 (Marshall & Kumacz, 1988). Thus, PGG_2 must be released by the cyclooxygenase and then become bound to the peroxidase site. A synergistic interaction exists between the two activities that are present in the same molecule. Cyclooxygenase requires hydroperoxide while peroxidase activity removes hydroperoxide. Both activities require one haem per subunit for full catalytic activity (Hsuanyn & Dunford, 1992).

The human PGHS-2 gene is 8.3 kb in length, similar to the PGHS-2 gene of mouse and chicken but smaller than the 22 kb human PGHS-1 gene. The sequence of the cDNA of both PGHS-1 and –2 shows 60% homology although the length of the two mRNAs is different, 2.8 kb for PGHS-1 vs. 4.5 kb for PGHS-2. The difference is due to the long 3`-untranslated region of PGHS-2, which confers instability to the mRNA, thus enhancing its degradation (Bazan *et al.*, 1996). The mRNA of PGHS may undergo alternative splicing resulting in a shorter transcript than the original. Levels of the 4.5 kb mRNA rapidly increase in human monocytes stimulated with pro-inflammatory agents, such as interleukin 1 β (IL1β), and decrease in monocytes treated with anti-inflammatory agents such as dexamethsone (Piomelli, 1993), supporting the hypothesis that PGHS-2 produces prostaglandins involved in inflammation (Chulada *et al.*, 1996).

Both catalytic activities of the synthase require a haem group (Granstrom & Kumlin, 1987). It is known that the cyclooxygenase activity in PGHS is subject to suicide inactivation, due to the production of free radicals through the oxygenation of AA (Hsuanyu & Dunford, 1992). Adding reducing agents such as phenol and glutathion usually protects the enzyme from self-catalyzed inactivation, most likely by removing the

free radicals resulting from the cyclooxygenase reaction (Granstrom & Kumlin, 1987). The self-inactivation is often seen when using high concentration of the substrate AA, possibly due to the acceleration of the cyclooxygenase reaction without suitable time for the peroxidase reaction to take place (Hsuanyu & Dunford, 1992; Wu *et al.*, 1999). There is some evidence that the self-inactivation of cyclooxygenase and also of lipoxygenase is favored under conditions where there is high liberation of free fatty acids, such as damage to the cells (Granstrom & Kumlin, 1987).

From his study comparing the effects of AA concentrations on the activity of PGHS-1 and –2, Swinney *et al.*(1997) concluded that the production of PGs proceeds through transcriptional regulation of PGHS-2 and allosteric regulation of PGHS-1 with a cooperative activation of the latter in response to AA concentrations. This might illustrate the higher activity of PGHS-2 than PGHS-1 when there is a limited source of AA in cells.

The non-steroidal anti-inflammatory drugs (NSAIDs) have been used for decades as inhibitors of inflammation. These inhibit the production of the potent inflammatory mediators, the prostaglandins, by interacting with PGHSs mostly by competing with the substrate. Among the best known, indomethacin inhibits PGs biosynthesis in a time-dependent and reversible manner (Rome & Lands, 1975). Aspirin, on the other hand, is unique in that it is the only NSAID known to covalently modify PGHS (Meade *et al.*, 1993). It seems that the aspirin modification in the hydrophobic channel of the enzyme blocks the substrate access to the active site through steric hindrance (Bazan *et al.*, 1996). Classical NSAIDs inhibit only the COX activity of both isoforms, leaving the peroxidase activity unaffected.

Recently, a new generation of PGHS-2 selective inhibitors having little or no effect on

PGHS-1 are promising as anti-inflammatory drugs that reserve the physiological action of constitutive PGs production by PGHS-1. Celecoxib and refecoxib are examples of selective PGHS-2 inhibitors.

I-I-III Phospholipase A_2 (PLA_2), Incorporation, and Remodeling Enzymes

The availability of AA is the rate limiting step in the biosynthesis of eicosanoids (Smith, 1989; Irvine, 1982; Tessier *et al.*, 1996). In the majority of cell systems membranous phospholipids are the principle source of AA for eicosanoid synthesis (Chilton *et al.*, 1987). The incorporation of AA into cells and its release before metabolism by target enzymes are well-controlled events. Several enzymes participate in regulating the amount of free AA in cells. Among these, phospholipase A_2 (PLA_2) [EC.3.1.1.4] is considered the main route for the release of AA due to its selectivity toward AA-containing phospholipids in membranes (Piomelli, 1993).

Several isoforms of PLA_2 have been characterized with differences in the primary structure, cellular localization, calcium requirement for activation and substrate specificity (Mayer *et al*, 1993). According to the calcium requirement, three major isoforms have been identified. A cytosolic PLA_2 ($cPLA_2$) isoenzyme that occurs as a 85 KDa protein with a calcium dependency in the micromolar range, a more abundant 14 KDa secretory isoform ($sPLA_2$) with calcium dependency in the millimolar range, and a 40 KDa calcium independent isoform ($iPLA_2$)(Dennis, 1994; Tessier *et al.*, 1996). The activation of the calcium dependent PLA_2 isoforms results from the elevation in calcium concentration in the cytoplasm derived from either Ca^{2+} influxes from the extracellular space or Ca^{2+} release from intracellular stores.

The cytosolic $cPLA_2$ requires micromolar Ca^{2+} concentration for translocation to membranes (Lin *et al.*, 1993), and possesses high specificity for arachidonyl group at the sn-2 position of phospholipids (Dennis, 1997). $cPLA_2$ activity is directly regulated by phosphorylation via the mitogen-activated protein kinase (MAPK) that in turn is activated by phosphorylation via the Ca^{2+} -activated protein kinase C (PKC) pathway (Golfman *et al*, 1999). On the other hand, $sPLA_2$ requires millimolar Ca^{2+} concentration for maximum activity and shows little or no selectivity toward the chain length or the number of unsaturated bonds of the acyl group at the sn-2 position (Dennis, 1997).

The esterified and the free AA levels in cells are controlled by a fine-tuned phospholipids deacylation-reacylation cycle in which PLA_2 plays a dominant role (Golfman *et al*, 1999). The PLA_2 mediated hydrolysis of PL results in the production of free fatty acid and a lysophopholipid acceptor that is rapidly re-esterified with another fatty acid by a coenzyme A (CoA)-dependent acyl transferase (Chilton *et al.*, 1987). The substrate of the latter enzyme is a CoA activated form of AA, a product of arachidonyl-CoA synthase. Both arachidonyl-CoA synthase and CoA-dependent lysophosphatidyl acyl transferase (LAT) are considered very active enzymes and do not appear to limit AA incorporation into phospholipids (Balsinde & Dennis, 1996).

In resting cells, the availability of lysophospholipid acceptors provided by a PLA_2-like activity is thought to be the rate limiting factor for the incorporation of AA (Daniele *et al.*, 1999). The PLA_2-like activity in resting cells seems to be independent of Ca^{2+} concentration, $iPLA_2$, and might act as a housekeeping enzyme for AA incorporation and remodeling (Ramanadham *et al.*, 1999). However, during cell activation the PLA_2-like

activity seems to be Ca^{2+} dependent resulting in the formation of lysophospholipids that serves as a substrate for CoA-independent transacylase, to drive arachidonyl-phospholipid remodeling (Daniele *et al.*, 1999).

A second major membrane storage site for AA is the triglyceride fraction where the diacylglycerol is the acceptor for the arachidonyl-CoA which is acylated via the enzyme diacylglycerol acyl transferase (DAT) [EC.2.3.1.20].

Placement in either phospholipid or triglyceride fractions is dependent on both the concentration of the free exogenous AA and the time of incorporation. Balsinde & Dennis (1996) found that the incorporation of micromolar quantities of [^{3}H] AA into phospholipids preceeded that into the triglyceride fraction, even though a major portion of AA eventually became incorporated into TG. Other studies indicated increasing levels of arachidonyl-triglyceride using high media concentrations of exogenous AA (Banerjee and Rosenthal, 1985). On the other hand, when a nanomolar concentration of AA was used, no radioactivity was detected in TG. Thus, the phospholipids constitute the major pool of AA and the incorporation of AA into TG portion may start shortly after the saturation of phospholipids using micromolar concentrations of AA (Balsinde & Dennis, 1996).

In the P388 D1 macrophage cell line, AA was first incorporated into phosphatidylcholine (PC) followed by a rapid transfer to phosphatidylethanolamine PE (Balsinde & Dennis, 1996). Wong *et al.* (1997) reported enhanced release of AA when human endothelial cells were incubated with lyso-PC in a time- and concentration-dependent manner. It was shown that lyso-PC increases intracellular calcium levels and stimulates protein kinase C PKC that activates the $cPLA_2$ indirectly through a

phosphorylation cascade (Golfman *et al.*, 1999).

Thus, the incorporation, release and remodeling of arachidonate in cells constitute a complex process by which the concentration of free AA is strictly controlled.

I-I-IV Cross Talk between PLA_2 and PGHS Isoforms.

As one may conclude, the AA movement within the cell after its uptake by the membrane acceptors is a dynamic entity, characterized by rigorous control of release and metabolism of the fatty acid.

The major rate limiting steps in AA metabolism via the cyclooxygenase pathway are mediated by four enzymes: ($sPLA_2$, $cPLA_2$, PGHS-1 and –2). Several studies were done to elucidate the coupling of these enzymes to the final product of the cascade, the prostaglandins (Balsinde *et al.*, 1999).

In most cell types, arachidonate added to the culture medium is rapidly incorporated into membrane phospholipids. After this, a short-term increase in PGE_2 production is observed due to conversion of the incorporated and then released free fatty acid by the constitutive enzyme, PGHS-1. A second and a delayed, though higher, PGE_2 production occurs after increased PLA_2 and PGHS-2 expression (Tessier *et al.*, 1996).

Reddy and Hershman (1996) proposed two independent pathways for stimulating prostaglandin synthesis: a secretory phospholipase A_2-mediated, PGHS-1-dependent transcellular and a cytosolic phospholipase A_2–mediated, PGHS-2-dependent intracellular pathways. This model is consistent with the hypothesis that PGHS-1 preferentially utilizes exogenous AA, while PGHS-2 prefers endogenous AA.

The lag-phase in prostanoid production observed after addition of exogenous AA

could be the result of a dynamic AA redistribution among cellular phospholipids, which usually takes hours, bearing in mind that PGHS-1 can only utilize AA liberated from cellular membranes (Gonchar *et al.*, 1999).

Recently, many studies were conducted to illustrate the cross talk between $sPLA_2$, $cPLA_2$ and PGHS-2 that mediates the inflammation-related delayed response of PGE_2 production (Murakami *et al.*, 1997; Balsinde *et al.*, 1999; Chulada *et al.*, 1997). In contrast to the immediate PG production which is regulated through allosteric activation of PGHS-1, the delayed PGs generation, which may requires several hours after stimulation with cytokines and growth factors, is generally regulated through transcriptional regulation of PGHS-2 and requires *de novo* protein synthesis (Swinney *et al.*, 1997).

In their study on one cell line genetically deficient in $sPLA_2$, Murakami *et al.*(1997) reported that $cPLA_2$ is responsible for initiating PGHS-2-dependent delayed PGE_2 production. $sPLA_2$ enhanced PGE_2 generation by increasing $cPLA_2$ and PGHS-2 expression mediated by the endogenous PGE_2 which acted as an amplifier of the delayed PG generation. The conclusion is that $sPLA_2$ hydrolyzes plasma membrane PL, leading to the generation of a small amount of PGE_2 which amplifies the $cPLA_2$/PGHS-2-dependent pathway. Chulada & Langenbach, (1996), on the other hand demonstrated that once activated, the $cPLA_2$ becomes associated with the nuclear membrane and modulate PGHS-2 activity in rat basophilic leukemia cells.

The kinetics of prostanoid synthesis depends on the source of AA, the irreversible inactivation of PGHS, prostanoid metabolism, incorporation of exogenous AA into membranes and finally the release of endogenous AA to the target enzymes (Gonchar *et*

al., 1999).

Aside from being the substrate for PG biosynthesis, AA also has a number of cellular functions. It can affect intracellular concentration of Ca^{2+}, leukocyte adhesion and several protein functions (Swinney *et al.*, 1997). It has been recently hypothesized that AA up-regulates the expression of $sPLA_2$ and PGHS-2 (Gonchar *et al.*, 1999). On the contrary, PGE_2 was shown to have a negative feedback effect on PGs biosynthesis in uterine stromal cells (Tessier *et al.*, 1996).

In conclusion, PG biosynthesis, at least in mammalian cells, is rigorously controlled via several pathways, sometimes unique to the cell type. This precise control is necessary to provide the cell with the adequately required levels of PGs that are considered one of the most potent biochemical effectors in the body.

I-II Arachidonate and Prostaglandins Metabolism in Ticks

I-II-I The Biological Significance of Ticks

The importance of ticks comes from the variety of pathogens they transmit to the host which include protozoa, rickettsiae, bacteria, viruses, fungi and filariae (Sonenshine, 1991). Several human diseases are transmitted by ticks ranking them second only to mosquitoes in their infectious transmission. Lyme disease and Rocky Mountain Spotted Fever are two major human diseases transmitted by ticks (Bowman *et al.*, 1996).

Ticks are unusual amongst arthropods in their ability to continuously feed on the host for a relatively long period of time, which may be as long as two weeks in the case of the female lone star tick *Amblyomma americanum*. The feeding pattern of ticks necessitates

counteracting the host immune and inflammatory response to tick feeding. The great efficiency exhibited by ticks is assumed to be facilitated by pharmacologically active compounds secreted in their saliva. Salivary factors include anti-coagulants (Gordon & Allen, 1991), apyrase that prevents platelet aggregation (Riberio *et al.*, 1985) and prostaglandins (Riberio *et al.*, 1985, 1992). The high level of PGs in tick saliva is assumed to be immunosuppressive, vasodilatory and anti-inflammatory (Bowman *et al.*, 1996). In addition, protein factors in tick saliva have been reported to be immunosuppressive (Wikel, 1996). The concentration of PGE_2 in dopamine induced-saliva of *A. americanum* is extremely high, about 2200 ng/ml (unpublished data) compared to 2 pg/ml of PGs in the peripheral plasma of humans.

I-II-II Changes During the Feeding of the Female Tick, *A. americanum.*

Ticks can survive several months without taking a blood meal (Needham and Teel, 1991). Upon attachment to a host, the female tick enters a slow phase of feeding lasting 8-14 days when its weight increases to approximately 300 mg followed by a rapid phase lasting 12-24 hrs during which the replete weight increases to 500-1000 mg (Sauer *et al.*, 1979). The male tick feeds intermittently and increases its weight after feeding remains small relatively little compared to the female tick (Sauer & Essenberg, 1984).

Mating stimulates feeding in female ticks and results in an increase in the amount, but not the number, of proteins synthesized in the salivary glands, as observed on SDS PAGE gels. Without mating, the weight of female tick usually does not increase above approximately 35 mg (McSwain *et al,* 1982).

The salivary glands consist of three types of acini; Type I are involved in the uptake of

water vapor and changes little upon tick attachment and feeding. Type II and III acini undergo major increases in size and mass and secrete numerous products needed for feeding (Fawcett *et al.*, 1986). Sauer *et al.* (1979) reported that the salivary glands gain competence to secrete fluid only upon the onset of feeding. After the first 4-7 days of feeding, the female salivary glands enlarge by more than 25-fold until the weight of females reaches 100-250 mg (Shipley *et al*, 1993). No further increase in salivary gland mass is observed beyond that weight. Shelby *et al.* (1987) reported a dramatic increase in RNA synthesis at the onset of tick feeding. Increased temperature upon attachment to the host, blood meal constituents and the physiology of females after mating are probably the major causes of the observed cytological changes observed in the salivary glands after the female ticks attach (Shelby *et al.*, 1987).

I-II-III Arachidonate Metabolism in Tick Salivary Glands

During tick feeding, the amount of fatty acids in both phospholipids and neutral lipid fractions increase substantially and reach a peak at a female weight of 100-250 mg. Arachidonic acid constitutes about 1.3% of all fatty acids in salivary glands of unfed ticks. This percent increases up to 8% in salivary glands in rapidly feeding female ticks. The amount of AA in each gland increases 40-fold during feeding reaching 1.64 µg /gland in rapidly feeding tick (Shipley *et al.*, 1993). Using several labeled fatty acids, Bowman *et al*, (1995a) proved that AA in tick salivary glands is sequestered from the host blood meal and is not synthesized in the tick.

Arachidonate uptake into cells might occur via two independent processes: one is active and facilitated, involving a plasma membrane protein and Na^+-fatty acid

cotransport system (Stremmel, 1987). The second is a physiochemical process involving spontaneous diffusion across the plasma membrane (Noy *et al.*, 1986).

Bowman *et al*, (1995b) showed that the uptake of AA might favor an active facilitated process through which there is a competition between the labeled and the unlabeled fatty acid. The amount of [^{3}H]AA incorporated by the salivary glands decreased from 40% at 10^{-8} M unlabeled AA to 2% at $5x10^{-3}$ M AA during one hour of incubation. Energy was assumed to play an active role in the uptake process.

A major portion of AA in both unfed and virgin female salivary glands is in the triglyceride fraction and serves as a source of metabolic energy in ticks that undergo prolonged periods without feeding (Shipley *et al.*, 1993).

In contrast, the phospholipid fraction in fed females contains most of the AA which is esterified to the sn-2 position of salivary glands phospholipids, a common feature of prostanoid producing tissues (MacDonald and Sprecher, 1991).

When the medium concentration of AA was raised above 10^{-5}M, the portion of [^{3}H]AA in the phospholipids decreased from 80% to 55% with a concomitant increase in [^{3}H]AA in the triglyceride fraction (Bowman *et al.*, 1995c).

Studies with isolated salivary glands demonstrated that the incorporation of AA into PC and PE occurred via the Land pathway involving an LAT enzyme [EC.2.3.1.23] (Bowman *et al.*, 1995c; Shipley *et al.*, 1994). It was hypothesized that tick salivary gland has an LAT enzyme with higher affinity for arachidonyl-CoA than the DAT [EC.2.3.1.20] which esterifies AA into TG. Thus, the esterification of AA into TG begins only after the LAT enzyme is saturated with high amounts of exogenous AA (Bowman *et al.*, 1995c).

Bowman *et al.* (1995c) also showed that the incorporation of AA into PL occurs initially into the PC followed by transfer to PE via a transacylase. No [^{3}H]AA was detected in phophatidylinositol (PI) suggesting that phosphoinositide phospholipase C (PLC) has no role in releasing endogenous AA (Bowman *et al.*, 1995d). However, a considerable variation in AA distribution between PL portions in tick salivary glands was noted and assumed to be the result of the feeding pattern of the individual tick.

The salivary glands of *A. americanum* have a 54 kDa, micromolar Ca^{2+} sensitive PLA_2 activity capable of releasing endogenous AA in response to Ca^{2+} ionophore A23187 up to 20 μM (Bowman *et al.*, 1994). Dopamine also has a stimulatory effect on the AA release mediated via the opening of voltage-dependent calcium channel during dopamine stimulation (Bowman *et al.*, 1995d).

A substantial difference was noted in the amount of free [^{3}H]AA (175%) and free endogenous AA (37%) in glands stimulated with A23187 (Bowman *et al.*, 1995d). Thus, much of the endogenous AA is not available for release by PLA_2 as compared to newly assimilated [^{3}H]AA.

Another possible isoform of tick PLA_2 was detected in the saliva with a molecular weight of 55 kDa and Ca^{2+} requirement in the submicromolar range. Greater than 100% of tick saliva PLA_2 activity was recovered by purification via size exclusion chromatography. In contrast, the activity of the salivary gland PLA_2 was substantially reduced after fractionating the salivary gland homogenate (Bowman *et al.*, 1997).

I-II-IV Evidence of PG Biosynthesis in Ticks.

Several points of evidence indicate that the salivary gland of ticks is a prostaglandin

producing organ (Bowman *et al.*, 1995b). First is the sequestration of a large amount of AA through feeding. As previously mentioned, the distribution of AA in PL resembles what is found in prostaglandin producing tissues (Bowman *et al.*, 1995a). Moreover, the saliva excreted from tick salivary glands contains extremely high concentration of PGE_2 that reaches in *A. americanum* about (2200 ng/ml) and in *I. dammini* about (100 ng/ml) (Ribeiro *et al.*, 1992; Inokuma *et al.*, 1994). This amount of PGE_2 is much larger than the vertebrate inflammatory exudates that ranges from 0.5-20 ng/ml (Monacada *et al.*, 1978). The high PGE_2 content in the saliva may prevent platelet aggregation and cause vasodilation, both necessary to maintain a suitable blood supply to the tick mouth-parts. PGE_2 also inhibits mast cell degranulation that minimizes the host inflammatory response against the tick (Shipley *et al.*, 1993).

In cultured salivary glands of *Hyalomma anatolicum excavatum,* the amount of both PGE_2 and $PGF_{2\alpha}$ increased during a 72 h incubation which indicates their capability of prostaglandin biosynthesis (Shemesh *et al.*, 1979). The existence of both the substrate and the products may suggest that the tick has evolved to produce PGs that are important in its parasitic life.

Bowman *et al.* (1995b) have shown that the whole female lone star tick is capable of *in vivo* PGs biosynthesis. The dopamine-induced saliva from [^{3}H]AA fed ticks contained radiolabeled PGE_2 and $PGF_{2\alpha}$. However, no PG biosynthesis activity was detected in the homogenate of salivary glands treated with [^{3}H] AA using standard conditions for *in vitro* detection of PGHS activity in isolated tissues. No endogenous inhibitor of PGs biosynthesis was in the salivary gland homogenate nor did the tissues rapidly metabolize [^{3}H] prostaglandin.

Using [^{3}H]AA, Pedibhotla *et al.*, (1995) demonstrated that the internal tissues of the whole female tick of *A. americanum* and the salivary glands (Pedibhotla *et al.*, 1997) were capable of producing small amounts of four different prostaglandins: PGA_2/B_2, PGD_2, PGE_2 and $PGF_{2\alpha}$. PGs synthesis was inhibited dose-dependently by both indomethacin and naproxen.

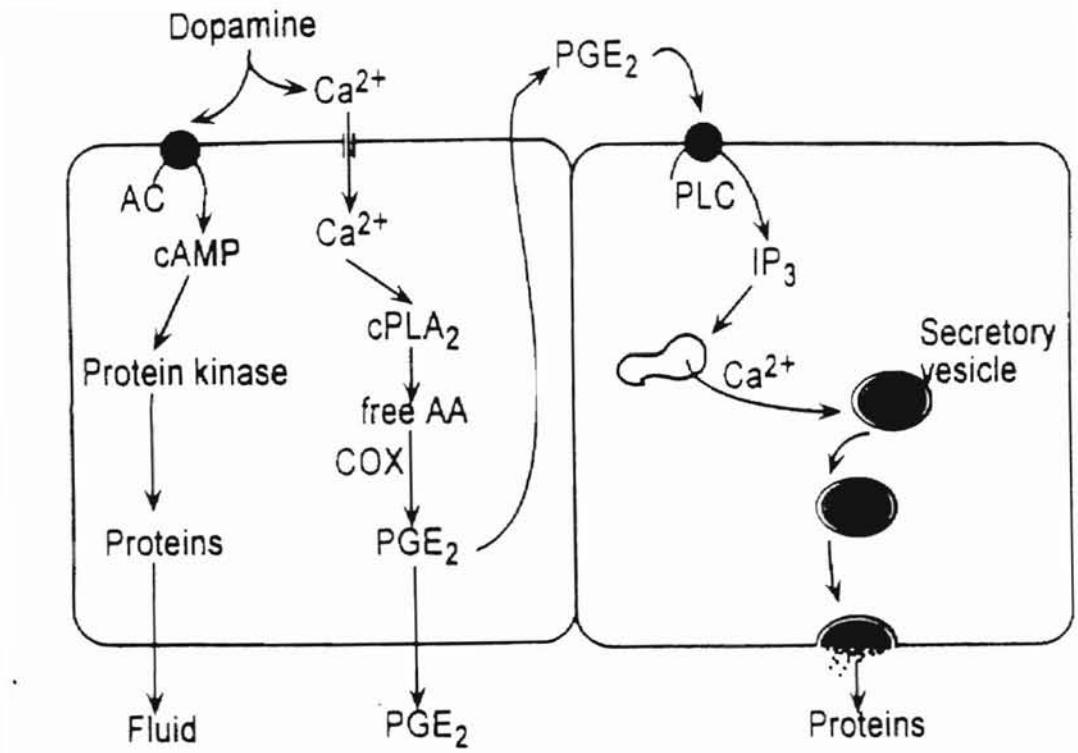
PG biosynthesis detected in the last two studies was much lower than expected, about 0.35 pmol of PGs/mg protein/hour, in comparison to the high molar concentration of PGs in the saliva.

A PGE_2 receptor in the salivary glands of female *A. americanum* was identified that increased the intracellular levels of inositol triphosphate (IP_3) via the activation of phosphoinositide PLC signaling pathway. The consequence of IP_3 elevation is the mobilization of Ca^{2+} from intracellular stores. The signaling pathway initiated by PGE_2 was shown to stimulate secretion of anticoagulant protein into tick saliva (Qian *et al.*, 1998).

The proposed transduction pathway concerning the PGE_2 metabolism and biological role in tick salivary glands is shown in Fig.1.2 (Qian *et al.*, 1998).

Fig 1.2 Proposed mechanism of PGE_2 synthesis and effect in tick salivary glands.

PGE_2 is synthesized by PGHS utilizing free AA released by $cPLA_2$. The produced PGE_2 is either secreted into saliva, where it has a potent anti-inflammatory and immunosuppressive effect, or acts as a paracrine effector that results in an increase in intracellular Ca^{2+}, which activates secretion of biologically active proteins.


Dopamine
Ca^{2+}
AC
cAMP
Protein kinase
Proteins
Fluid
Ca^{2+}
$cPLA_2$
free AA
COX
PGE_2
PGE_2
PGE_2
PLC
IP_3
Ca^{2+}
Secretory vesicle
Proteins

Objective and Specific Aims

Ticks are of major medical and veterinary importance. They mediate the transmission of several serious diseases of both humans and livestock. The unique feeding pattern of ticks facilitates the transmission of tick-born pathogens and is a major potential target for pharmacological interference to control ticks.

Prostaglandins in tick saliva may play a substantial role in maintaining the necessary environment at the tick / host interface for the continuity of tick feeding. This may include immunosuppressive, anti-inflammatory and vasodilatory effects in the host, in addition to inducing secretion of several major proteins, such as anti-coagulants, from the salivary glands into the saliva (Bowman *et al*, 1997 and Qian *et al*, 1998).

Several recent pieces of evidence suggest an ability of the lone star tick to synthesize prostaglandins (Bowman *et al.*, 1995d; Pedibhotla *et al.*, 1995;1997), but the apparent synthesis by isolated glands was unexpectedly low or undetectable in these studies.

Therefore, the objective of this study was to re-investigate the *in vitro* capability of the isolated salivary glands of the female lone star tick, *Amblyomma americanum*, to synthesize PGE_2.

My specific aims were to:

1. Determine PGE_2 biosynthesis at various concentrations of exogenous arachidonic acid.
2. Determine the turnover rate of labeled arachidonate into labeled products.
3. Investigate the role of indomethacin in inhibiting PGE_2 biosynthesis.
4. Clone the gene of PGHS enzyme responsible for PGs biosynthesis using the total RNA of tick salivary glands.

CHAPTER II

MATERIALS AND METHODS

II-I Prostaglandin E_2 Biosynthesis in the Salivary Glands of the Female Lone Star Tick, *Amblyomma americanum*

Chemicals

The following chemicals were obtained from Sigma Chemical Company (St.Louis, MO): hemoglobin, glutathione, p-amino benzamidine, hematin, indomethacin, calcium ionophore A23187, dopamine, TC-199 tissue culture medium (M-0393), CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfate), Sigmacoat, and Phenol. ETYA (5,8,11,14-Eicosatetraynoic acid) was purchased from Biomol Inc (Biomol, PA, USA).

Arachidonic acid [5,6,8,9,11,12,14,15-^{3}H(N)] (60-100 Ci/mmol) and prostaglandin E_2 [5,6,8,11,12,14,15-^{3}H(N)]-(100-200 Ci/mmol) were obtained from NEN Life Science Products Inc. (Boston, MA). Unlabeled prostaglandin standards and arachidonic acid were obtained from Cayman Chemical Company (Ann Arbor, MI). Ram seminal vesicles microsomes were obtained from Oxford Biochemical Research Inc (MI, USA).

Tick Rearing

Adult, partially fed female lone star ticks, *Amblyomma americanum* (L.), weighing 50-150 mg were used in all experiments. Ticks were reared at Oklahoma State University's Central Tick Rearing Facility, according to the methods of Patrick and Hair (1975).

Salivary Glands Preparations

Salivary glands were dissected and cleaned of other tick tissues under a dissecting microscope in ice-cold TC-199 containing 20 mM morpholinopropanesulfonic acid (MOPS) buffer (pH 7.0). In some experiments right and left salivary glands were used as control and experimental groups, respectively. Glands were transferred to pre-siliconized microfuge tubes and used within one hour of dissection. Unless otherwise indicated, most incubations were done in TC-199 MOPS buffer containing various drugs, and arachidonic acid concentrations, with the solvent of these drugs added to the control tissues. Salivary gland homogenate was prepared via sonication in TC-199 MOPS buffer containing 0.05% (W/V) p-amino-bezamidine and 0.1 mM ethylenediaminetetraacetic acid (EDTA). Salivary glands microsomes were prepared by homogenizing the tissues in ice-cold 10 mM Tris-HCl, 0.25 M sucrose, 10 mM $MgCl_2$, 0.1 mM EDTA, pH 7.2 containing 0.05% (w/v) p-amino-benzamindine. The homogenate was centrifuged at 900 g for 10 min, and the supernatant was centrifuged at 11,500 g for 10 min. The 11,500 g supernatant was re-centrifuged at 100,000 g for 60 min and the resultant microsome-enriched pellet was reconstituted in 200 µl of incubation buffer and kept on ice for the PG assay. Aliquots of the reconstituted pellet were added to 500 µl buffer, mixed and the protein concentrations were measured. In some experiments, further enrichment of the enzyme fraction was tried using solubilizing buffer. The salivary gland microsome-enriched pellet was incubated on ice for 60 min in the solubilizing buffer (10 mM Tris pH 8 containing 1% CHAPS and 0.5 mM EDTA). The solubilized microsomes were centifuged again for 60 min at 100,000 g. The resulting supernatant was loaded onto

Centricon 3 columns (Amicon Inc, Beverly, MA), centrifuged at 7000 g for 30 min, and washed twice with 300 µl of 10 mM Tris buffer pH 8. Finally, columns were inverted and centrifuged at 7000 g for 2 min and the recovered volume was kept on ice to measure protein concentrations and PG activity.

For the PG biosynthesis assay, a mixture of PGHS cofactors was added to the incubation medium for the final concentration of 0.4 µM hemoglobin, 5 mM glutathione, and 1 mM phenol.

Radioimmunoassay (RIA)

After incubation periods, an amount of 3% formic acid was added to the samples to adjust the pH (3-4), and the samples were sonicated twice each for 20 sec, centrifuged at 12000g for 5 min. A suitable volume of ethanol was added to the supernatant to a final concentration of 15% and the samples were extracted according to the method of Powell, (1982) using a 500 mg Sep-Pak C18 cartridge (Waters, Milford, MA, USA). Columns were prepared with ethanol and water, then samples were loaded on the columns and washed sequentially with water, 15% ethanol, petroleum ether, and petroleum ether/ chloroform (65:35). Finally the samples were eluted with ethyl acetate and dried under N_2. Samples were reconstituted in RIA buffer (0.01 M sodium-phosphate-buffered saline containing 0.1% BSA and 0.1% sodium azide). A quantity (0.1 ml) of sample or standard concentrations of PGE_2 in RIA buffer was incubated on ice with PGE_2 antiserum for 30 min. A quantity (0.1 ml) of buffer containing 5 pg of [^{3}H]PGE_2 (5500-6000 dpm) was added and the mixture was incubated for 60 min. Cold dextran (0.1%) coated and activated charcoal suspension (0.2 ml) was added and incubated in ice water for 10 min.

The tubes were centrifuged at 2000g at 4°C for 15 min, and the radioactivity of 800 µl of supernatant was measured by liquid scintillation counting by Scintisafe Econo2 (Fisher Chemical, NJ). Samples were diluted by RIA buffer until the values were in the range of 31-1000 pg.

Gas Chromatograph/ Mass Spectrometry (GC/MS)

Samples of salivary glands were extracted as for RIA. Dried samples were reconstituted and derivatized according to Barrow & Taylor, (1989) and Ngan & Toofan, (1991). The prostaglandins in the samples were sequentially derivatized using ethereal diazomethane, 2% methoxyamine-HCl in pyridine and BSTFA. Derivatized samples were reconstituted in 20 µl of 99% dodecane prior to splitless injection. Analysis was performed with a Hewlett-Packard GC model 6890 interfaced to an HP5973 Mass Selective Detector.

Separation was performed on a 30 m X 0.25 mm internal diameter, 0.25 µm film thickness, HP-5MS capillary column with oven parameters set at 200°C for 5 min, ramping to 290°C at 10°C/min and holding for 8 min. Analysis utilized both total ion scans and selective ion monitoring for the identification and quantification of PGE_2 using HP Chem station software and comparison with the same parameters for authentic PGE_2 standard.

A linear regression analysis was constructed using varying quantities of authentic PGE_2 standards. The r^2 were typically between 0.49 and 0.99. Authentic PGE_2 was subjected to the same extraction procedures and resulted in a 98% recovery. Natural samples were spiked with known amounts of authentic PGE_2 to check procedural

efficiency.

Thin Layer Chromatography (TLC)

Salivary gland samples containing labeled AA and its products were extracted after homogenization either directly with ethyl acetate, or after solubilizing the tissues using methylene dichloride and methanol according to Bligh & Dyer, (1959). After extraction, samples were dried under N_2 and reconstituted with 100 µl of ethyl acetate. Thin-layer chromatography plates 20 X 20 cm, 250 µm thickness channeled silica gel G with a preabsorbent zone were obtained from Analtech (Newark, DE). The solvent system used was chloroform-methanol- acetic acid-water (90:8:1:0.8, v/v/v/v) (Salmon & Flower, 1982). Chloroform and methanol were purchased from Fisher Scientific (Pittsburgh, PA). Authentic PGE_2 and AA standards with other individual lipids, in addition to [^{3}H]PGE_2 and [^{3}H]AA, were run with each sample. The detection of radioactivity was done either by radioscanning the TLC plate using Bioscan 2000 (Bioscan, Washington, DC), or by cutting bands along sample lanes and measuring the radioactivity by scintillation counting using liquid scintillation cocktail (BioCount, Research Product International CORP). Unlabeled lipid standards were recognized by subjecting the TLC plate to iodine vapor for 30 min and the R_f was calculated for each band relative to the solvent front.

Protein Assay

Protein concentration was determined by the method of Bradford (1976) with Bio-Rad protein assay dye using bovine serum albumin as the protein standard.

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Numbers of replicates are indicated in figure legends. Student's t test was used to test the significance of the differences between the control and experimental groups, with a $P<0.05$ considered as significant.

II-II Cloning a Gene for PGHS from Tick Salivary Glands

Extraction of Total RNA

Tick salivary glands were dissected out and carefully cleaned in ice-cold MOPS buffer pH 6.8, and then immediately immersed in liquid nitrogen. All tubes were sterile and RNase free.

The total RNA was extracted using Rapid Total RNA Isolation Kit (5`,3`, Inc, Boulder, CO). Tissues were homogenized in 1 ml guanidinium isothiocyanate using sterile syringes by pipetting the mixture up and down several times. The homogenate was transferred to a pre-spun phase lock gel tube, and 0.1 ml of 2.0 M sodium acetate, pH 4.0 added and mixed with the homogenate. One ml of H_2O-saturated phenol was added and mixed thoroughly with several inversions followed by addition and mixing of 0.3 ml of chloroform-isoamyl alcohol (49:1). After incubation on ice for 10 min, the mixture was centrifuged at 4,500 g (4°C) for 5 min. The aqueous phase was transferred to a fresh pre-spun PLG tube. A 1 ml of phenol-chloroform-isoamyl alcohol (50:49:1) mixture was added to the aqueous phase and mixed thoroughly. The mixture was centrifuged at 4,500 g (4°C) for 5 min. An equal volume of 100% isopropanol was added to the aqueous

phase, mixed, incubated at room temperature for 20-30 min, then centrifuged at 16,000 g for 30 min. The pellet was washed and centrifuged 2-3 min at 16,000 g three times with 5 ml of 70% ethanol. The pellet was finally washed once with 5 ml of 95% ethanol and the final pellet was dried at room temperature. The pellet was dissolved in 200 µl of RNase free water and the concentration of RNA was determined by measuring the O.D. of the sample with an average concentration of 0.682 µg/µl. The total RNA was aliquoted in several RNase free tubes and stored in -80°C until usage.

RT-PCR

The cDNA from tick salivary glands total RNA was prepared using Superscript Preamplification System for First Strand Synthesis (Life Technologies, Gaithersburg, MD). The first strand was prepared using oligo (dT) and Superscript II reverse transcriptase, according to the manufacturer's instructions.

Primers

Four degenerate primers were constructed according to conserved regions among mammalian cyclooxygenase 1 and 2 isoforms (sequences are not shown).

In addition, two degenerate primers were designed according to similar regions between cyclooxygenase isoforms and peroxidases from different invertebrates including *Anopheles* and *Drosophila* peroxidases.

The two primers are:

5` GA(C/T)GGI(A/T)(G/C)ITG(C/T)AA(C/T)AA(C/T)(C/T)TICA(A/G)AA 3`

5` (G/A)TAIGTIGCIT(G/A)ICC(G/A)TG(G/A)TCIC(G/T)ICCIC(G/T)(G/T)TG 3`

A polymerase chain reaction was done using PCR Reagent System (Life Technologies) including Taq DNA Polymerase. A PCR control of chloramphenicol acetyl transferase (CAT) whose cDNA was prepared from control CAT RNA using the first strand protocol was run concomitantly.

After setting up the PCR mixtures, samples were denatured for 2 min at 95°C, then for 1 min at 94°C. The annealing lasted for 1 min at 55°C, and the extension for 1 min at 72°C. The whole cycle following the denaturation step was repeated 28 times followed by final extension for 15 min at 72°C. The PCR products were kept on ice or frozen at -20°C until used.

The PCR products were run on 0.8 % agarose gel with 0.01% ethidium bromide in an electrophoresis chamber to separate DNA molecules. Gels were visualized under UV light and the bands of interest were cut. The DNA was extracted from the gel using QIA Quick Gel Extraction Kit (QIAGEN, Valencia, CA). The PCR product was further purified using QIA Quick PCR Purification Kit (QIAGEN). The concentration of DNA was measured by O.D. and the DNA was ligated into PCR 2.1-TOPO plasmid (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The ligation product was transformed into chemically competent One Shot strains of *E.coli* (Top 10, Invitrogen) by heat shock. After transformation, cells were first incubated in SOS medium for 30 min at 37°C, and then spread onto selective agar plate, with ampicillin, X-gal, and IPTG. Cells were incubated overnight at 37°C and were subject to blue-white screening. Several white colonies were selected and inoculated into 3 ml LB-ampicillin growth medium and incubated for 18 hours at 37°C. The DNA was extracted from cells using QIA Prep Spin Miniprep Kit (QIAGEN) and the DNA concentration was measured

by O.D.

The DNA samples were sent to the Core Facility in Biochemistry and Molecular Biology Dept. at OSU to be sequenced with the Perkin-Elmer Automated DNA Sequencing System using four ABI fluorescent dye-laeled dideoxy nucleotides. The amplification was done using M13 forward and reverse primers and Taq DNA polymerase. The products were run on polyacrylamide gel and the sequence was determined using a photomultiplier tube that detects the fluorescent light and converts it into electrical signals.

The DNA and Protein sequence matches were determined using Blastn and Blastx data search, respectively.

CHAPTER III

RESULTS

III-I Effect of AA concentrations on PGE_2 biosynthesis in tick salivary glands

Previous studies of PG synthesis in isolated tick salivary glands used concentrations of AA, 10 μM, similar to those used in mammalian tissues (Pedibhotla *et al.*, 1995; 1997; Bowman *et al.*, 1995). In contrast, I tested concentrations of AA from 1 μM up to 1 mM taking into account the high amount of endogenous AA in the salivary glands and the possibility that a high concentration of exogenous substrate may be required to see synthesis above in control tissues. There was no difference in PGE_2 synthesis measured by RIA between control and experimental groups of glands at exogenous AA concentrations less than 10 μM consistent with previous assays. However, when higher concentrations of AA were used, a dose-dependent increase in PGE_2 above control levels was observed (Fig. 3.1). The increased rate of PGE_2 synthesis was up to 50 ng / salivary gland / hour at 1 mM AA.

Even though the antibody used to measure PGE_2 in the RIA was supposed to be highly specific for PGE_2, the high amount of exogenous AA used in the assay might lead to an over-estimate of the amount of PGE_2 due to cross-reactivity of AA with PGE_2 antibodies. To test this possibility, the PGE_2 in several samples were measured with both RIA and GC/MS, since GC/MS is considered as the definitive method for positively identifying and measuring the reaction product. Table 3.1, shows the levels of PGE_2 measured by

both methods, and Fig. 3.2 shows the correlation between the two methods where the correlation coefficient was (0.81), with no significant difference between the two methods ($P>0.05$).

III-II Detection of the turnover of [^{3}H] AA into labeled PGE_2

The next step in confirming the capability of tick salivary glands to synthesize PGE_2 was to detect the labeled product starting with labeled substrate, [^{3}H]AA. Intact salivary glands were incubated for one hour at 37°C with 0.1 µCi [^{3}H]AA, and the labeled products were run on a TLC and radioactivity measured in scarped bands and detected via liquid scintillation counting. A radio-chromatogram, Fig.3.3, of chromatographed labeled AA and PGE_2 shows two major peaks; one corresponding to AA, at 9 cm, and the other to both [^{3}H]PGE_2 and unlabeled PGE_2 authentic standards, at 4 cm. The highest radioactivity in this peak was seen using 500 µM concentration of AA that was spiked with 0.1 µCi of [^{3}H]AA. Further, we tested the dose dependency of the production of [^{3}H]PGE_2 on the concentrations of the unlabeled AA. Figure 3.4 shows a dose-dependent increase in [^{3}H]PGE_2, measured in the bands that corresponded to the authentic PGE_2 standards, relative to the concentration of unlabeled exogenous AA. All concentrations above 10 µM of AA resulted in a significantly higher [^{3}H]PGE_2 relative to the control.

In one experiment we compared the fold change of the formed PGE_2 using the three methods of detection; RIA, GC/MS, and TLC as a function of increasing concentrations of exogenous AA (Fig.3.5). Right and left salivary glands were used for incubation with different amounts of AA with and without labeled AA, respectively. Samples using the

unlabeled AA were divided to measure PGE_2 levels by both RIA and GC/MS. The three methods correlated well except at the highest AA concentration, 1 mM, where the amount of PGE_2 formed was less than that when using 500 µM AA in both TLC and GC/MS, while the amount measured by RIA was higher using 1 mM AA. It is possible that some AA cross-reacts with PGE_2 antibodies in RIA at high concentration of the substrate. The highest AA used in subsequent dose-dependent and inhibition studies was 500 µM or less. Figure 3.6 a, b, and c represent the selective ion monitoring scan for PGE_2 using 0, 100, and 500 µM of exogenous AA, respectively.

III-III PGE_2 Biosynthesis in Tick Salivary Glands Microsomal Pellet

The presence of PGHS isoforms in the endoplasmic reticulum and the nuclear envelope locates them in tissue microsomes following ultracentrifugation in mammals (Chulada *et al.*, 1996). The tick salivary gland microsomal fraction was prepared using differential centrifugation. The 100,000 g microsomal pellet was resuspended and aliquots were incubated with different concentration of exogenous AA spiked with 0.1 µCi of [^{3}H]AA. Figure 3.7 represents a radio-chromatogram of salivary glands microsomal pellet incubated with 0 µM, 500 µM, and 1 mM AA. Without unlabeled AA no radioactivity was detected that corresponded to the R_f value of authentic PGE_2 standards. In contrast, the radioactivity was much greater in PGE_2-related bands when tissues were incubated with 500 µM AA.

There was a third peak that represents compounds slightly more polar than AA. These compounds might be either an oxidized form of the free AA or dehydration products of

PGE_2, PGA_2/B_2 , although more research is required to confirm this hypothesis. In the latter case, the amount of PGE_2 formed throughout the incubation with the labeled substrate might be more than indicated by the radioactivity at the R_f value of authentic PGE_2 standards. Alternatively, a lipoxygenase and cytochrom P_{450} product, 5-HETE (Kuhn & Borgraber, 1998) also matches the R_f of this peak leading to the speculation that either or both enzymes might exist in tick salivary glands.

The amount of PGE_2 synthesized by the tick salivary gland microsomal fraction was also affected by the concentration of the unlabeled substrate (Fig.3.8). However, only 500 µM AA led to a significant increase in PGE_2 levels measured by RIA relative to the control. In contrast to their effect on intact cells, neither 100 nor 250 µM AA led to significant increase in PGE_2 above the control level in the microsomal fraction.

Levels of PGE_2 were measured by GC/MS in relation to exogenous AA concentrations in intact salivary glands, the microsomal pellet, and salivary gland homogenates (Table 2.2). In one experiment, salivary glands were incubated in 5 ml TC-199 MOPS buffer for one hour at room temperature with agitation to wash out endogenous PGE_2 from the cells. The amount and the fold increase in PGE_2 levels in these cells were substantially less than PGE_2 levels in cells that were directly incubated with the substrate. In addition, the amount of PGE_2 in the microsomal pellet was much less than that in the salivary glands homogenate, while the fold increase in PGE_2 formed throughout the incubation with the substrate was higher in the microsomal pellet than in the homogenate (2.69 vs. 1.84), respectively.

Figure 3.9 shows a comparison of different radioscans of intact salivary glands,

homogenate, microsomal fraction, intact salivary glands that were frozen before use, and a scan of ram seminal vesicles used as a control for PGE_2 synthesis (a, b, c, d, and e, respectively). All samples contained 500 µM AA in addition to the labeled substrate and were incubated for one hour at 37°C. Both the homogenate and the frozen tissues show no peaks other than the peak for [^{3}H]AA. The ram seminal vesicles scan demonstrated 5 peaks; the major peak, peak 1, corresponds to the R_f value of authentic PGE_2. The microsomal fraction scan demonstrated one peak, peak 1, which also appeared in the scan of the ram vesicles, peak 3. This peak might be a prostaglandin A_2/B_2 that results from the dehydration of PGE_2. The same product was seen in the intact fresh salivary gland scan beside the AA peak and the peak that corresponds to TG standard. Moreover, we tested the efficiency of PGE_2 synthesis in all fractions resulting from differential centrifugation with no major difference between them (data not shown).

III-IV Inhibition of PGE_2 Biosynthesis in Tick Salivary Glands

The rate limiting step in PG biosynthesis is mediated via the enzyme prostaglandin H synthase (Dubois *et al*, 1998). Several non-steroidal anti-inflammatory drugs are potent inhibitors of the enzyme. Indomethacin is a time-dependent and reversible inhibitor known to affect both isoforms of mammalian PGHS (Rome & Lands, 1975). We tested the inhibitory effect of indomethacin on the tick salivary enzyme (Fig.3.10). The microsomal fractions of salivary glands were preincubated with varying concentrations of indomethacin for 30 min before a fixed amount of the substrate 250 µM was added, and the amount of PGE_2 formed during the one hour incubation with the substrate was

measured by subtracting the level of PGE_2 in the control group, without AA. Both 200 and 300 μM indomethacin significantly inhibited PGE_2 synthesis in the salivary gland microsomal fraction. The inhibition was dependent on indomethacin concentrations. In Table 3.3 we compared the PGE_2 levels in the microsomal fraction using 500 μM exogenous AA with or without the inhibitor measured by both RIA and GC/MS. When the inhibitor was added, both methods resulted in less PGE_2 than in the control when only 500 μM AA was added. Thus, indomethacin is capable of inhibiting PGE_2 synthesis in tick salivary gland.

The negative values that resulted when the inhibitor was added may indicate that even without adding an exogenous source of AA. PGE_2 synthesis occurs utilizing the endogenous substrate. This latter synthesis can also be inhibited by indomethacin.

III-V Time dependency of AA utilization in Tick Salivary Glands

The inhibition by indomethacin suggests that the salivary glands utilize endogenous sources of AA to produce PGE_2 during *in vitro* incubations. When two groups of 10 salivary glands each, right and left, were incubated at 37°C without adding exogenous AA, the amount of PGE_2 measured by GC/MS increased from 16.11 ng / sample after one hour incubation to 116.0 ng / sample after four hours. Both samples were extracted according to Bligh and Dyer, (1959) that retrieves all lipids in the sample. Thus, the increase indicates the endogenous synthesis of PGE_2.

The same conditions were applied on another group of 10 right and left salivary glands except for the addition of [^{3}H]AA to both groups and the detection of labeled

products on a TLC plate (Fig.3.11, a and b). The scans of 1 and 4 hours incubation with the labeled substrate show a substantial difference of radioactivity distribution between the two with major peaks after 4 hours incubation period. No major peak was seen corresponding to the PGE_2 standards (at 7-8 cm) although the peak at 10-12 cm, peak 4, was higher after the longer incubation. This peak might correspond to PGE_2 dehydration products. Two other major peaks, peak 1 and 6, corresponded well to PL and TG authentic standards, respectively. On the other hand, the time dependency of [^{3}H]AA metabolism within one hour incubation period showed no difference between 15, 30, and 60 min of incubation (data not shown).

The incorporation of AA into phospholipids is a time dependent process (Bowman *et al.*, 1995b). Intact salivary glands incubated with only the labeled AA demonstrated a major difference in both the free arachidonic acid and the one that was incorporated into phospholipid fraction (Fig.3.12 a,b). When the salivary glands were incubated for four hours, a major fraction of the free AA was incorporated into the PL fraction seen by the increase in the PL peak accompanied by a decrease in the peak of free AA. No major difference in TG peak was observed after the longer incubation.

III-VI Effectors of AA utilization in the Salivary Glands.

In an attempt to determine the effect of the high concentration of exogenous AA on AA metabolism, we preincubated the salivary glands with [^{3}H]AA for three hours and then added 500 µM exogenous AA to the incubation medium (Fig.3.13 a,b).

In addition, we tested the effect of 10^{-4} M dopamine and 10^{-4} M of calcium ionophore A23187, both known to activate PLA_2 and affect free levels of AA in tick salivary glands

(Bowman *et al.*, 1995c) (Fig.3.13 c,d). Compared to the control, the effect of unlabeled AA was seen mainly in the level of [^{3}H]AA in PL and TG peaks. In both fractions, the radioactivity decreased indicating the release of [^{3}H]AA mediated by the unlabeled AA. A similar pattern was seen using calcium ionophore known to cause the release of AA from PL by activating the hydrolysis of PL via PLA_2. Dopamine had no significant effect compared to the control within experimental conditions. When unlabeled AA was added with the [^{3}H] AA during the preincubation, no radioactivity was shown in the PL fraction and most of the radioactivity was in the form of the free AA (Fig.3.13, e).

When incubated with the salivary glands, indomethacin had no significant effect on the radioactivity distribution among the major peaks (data not shown).

III-VII The Characterization of a Peroxidase Gene in Tick Salivary Glands

Using BLAST search, all PCR product resulting from the amplification of salivary glands cDNAs using the cyclooxygenase primers had no similarity to either DNA or protein sequences of mammalian cyclooxygenases.

The amplification of tick salivary cDNA using peroxidase primers resulted in a 1.2 Kb DNA segment that had a protein similarity with several peroxidases (Table 3.4). Further work is required to clone the whole gene and determine the significance of its product to the tick physiology.

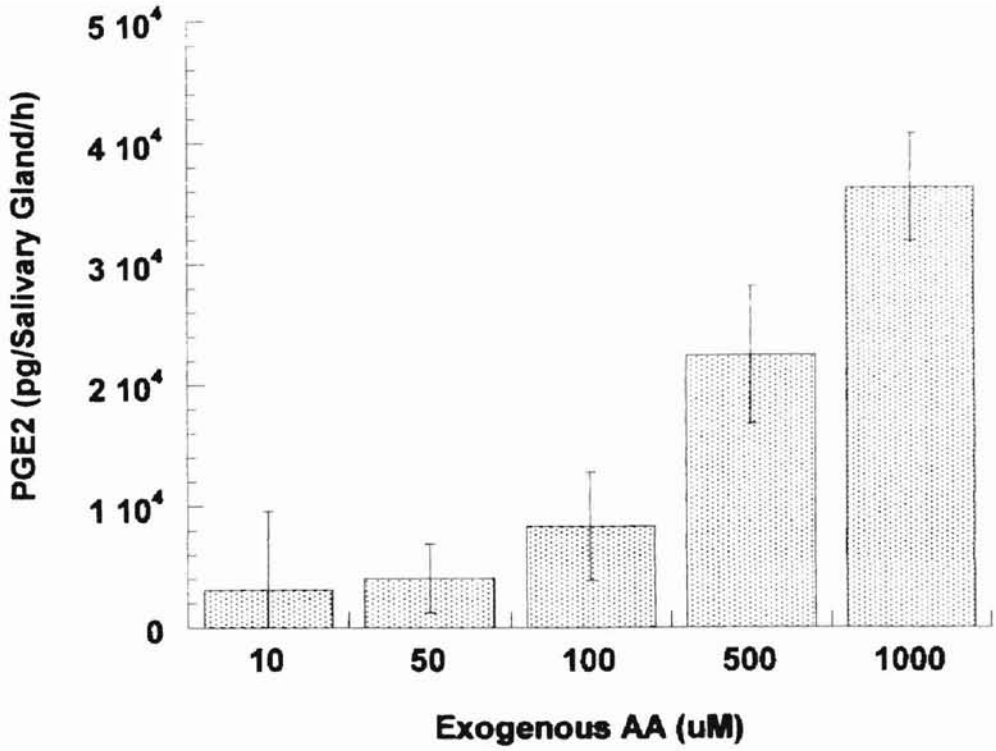


Fig. 3.1. Dose dependent increase in tick salivary gland PGE_2 levels incubated with increasing concentration of AA. Values are mean ± SEM, N=5. Data represent PGE_2 in experimental - control. Right and left salivary glands were used as experimental and control groups, respectively.

Samples	RIA	GC/MS
1	9.22	9.20
2	17.00	17.00
3	10.43	10.74
4	43.20	23.40
5	2.20	7.57
6	63.00	34.20
7	2.95	11.32
8	54.90	31.46
9	12.30	10.16
10	21.50	34.39
11	36.08	33.40
12	15.79	32.90
13	73.60	45.90
14	24.80	32.93
15	121.50	111.66
16	32.60	45.65
17	30.30	33.65
18	37.08	37.53
19	50.38	56.78
20	61.44	136.05
21	114.60	109.60
Average	39.76	41.21
STD	33.10	35.51
P value	0.76	
Correlation R2	0.81	

Table 3.1 A comparison of PGE_2 levels on the same samples (ng) in tick salivary glands between 21 samples measured by both RIA and GC/MS.

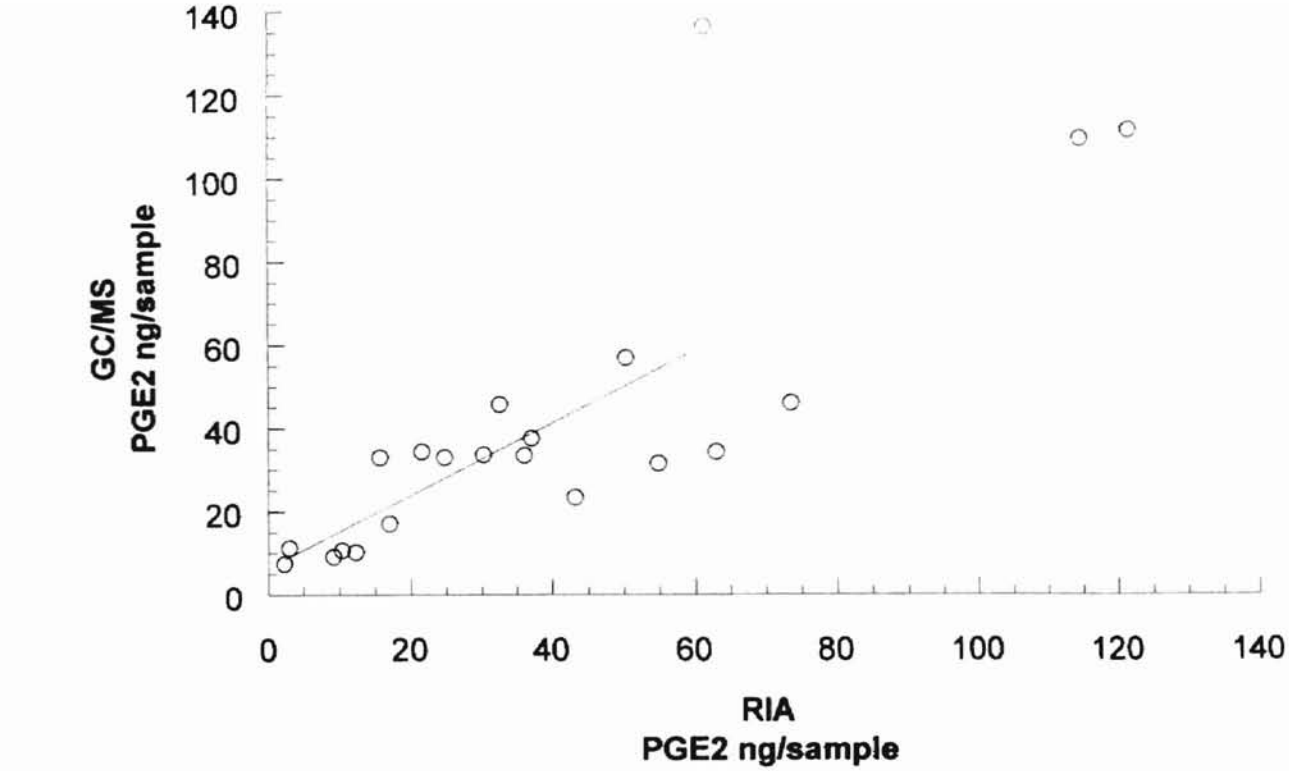


Fig.3.2 Correlation between PGE_2 levels in the same samples of tick salivary glands measured by both RIA and GC/MS. N= 21 samples, r^2=0.81.

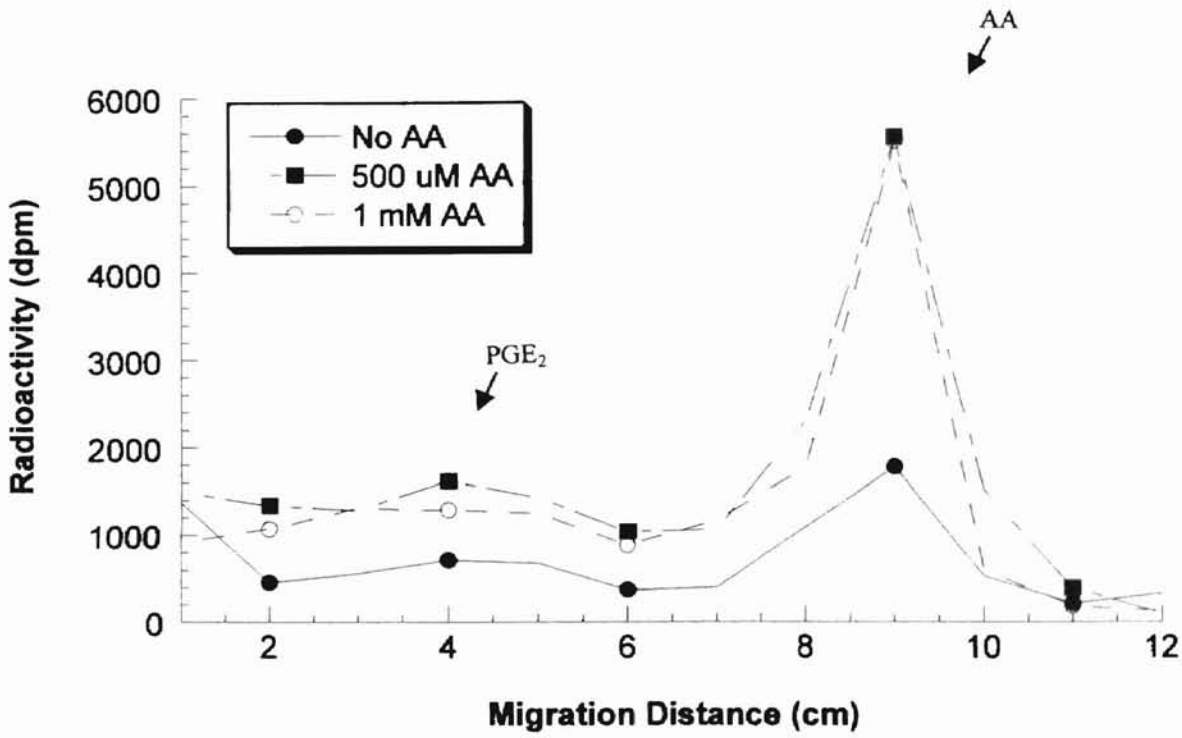


Fig.3.3 Thin layer radiochromatogram of homogenized and extracted whole tick salivary glands incubated with 0.1 μ Ci [^{3}H]AA and varying concentrations of AA. All bands of sample lanes were cut and the radioactivity was measured by liquid scintillation counting. Arrows indicate migration distances of authentic PGE_2 and AA.

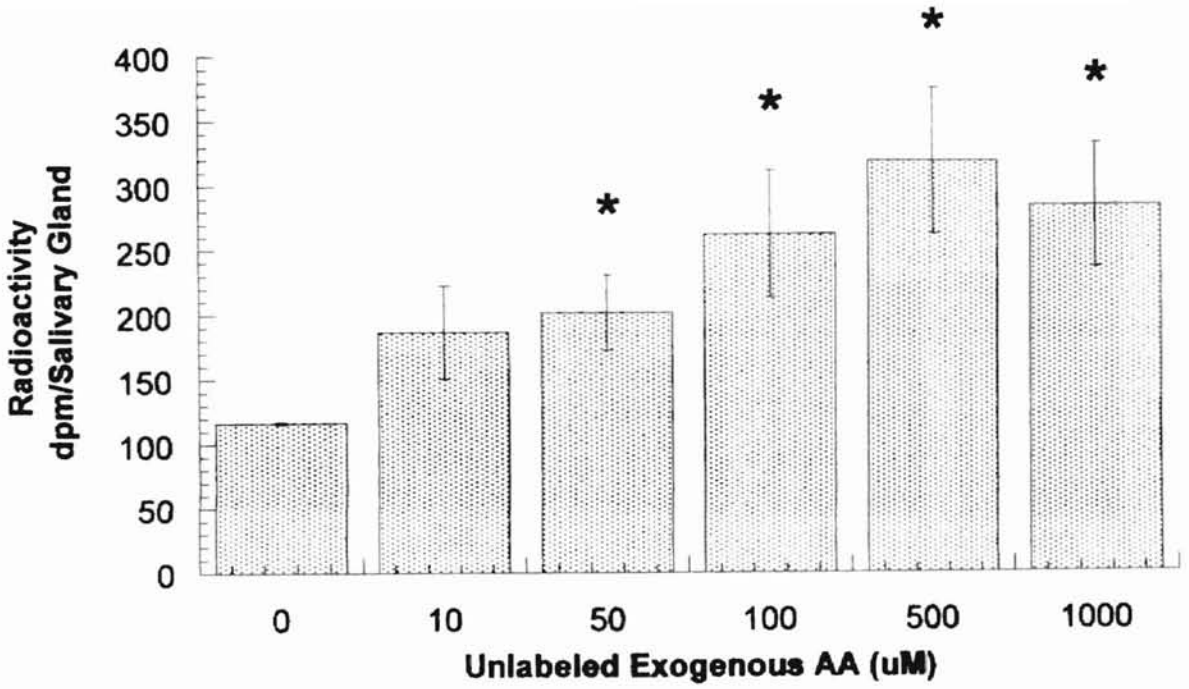


Fig.3.4 Increase in radioactivity measured on TLC plates co-migrating with band for authentic PGF_2 as a function of unlabeled exogenous AA concentrations during incubations of whole salivary glands. Data are mean ± SEM, N=5. * is significantly different from the control w/o AA.

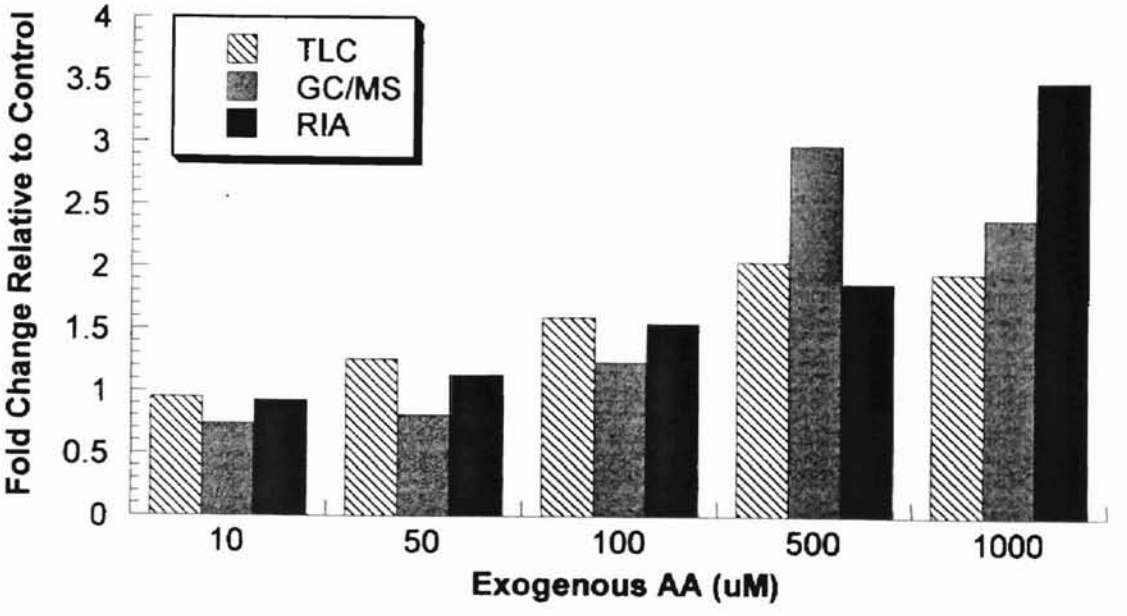
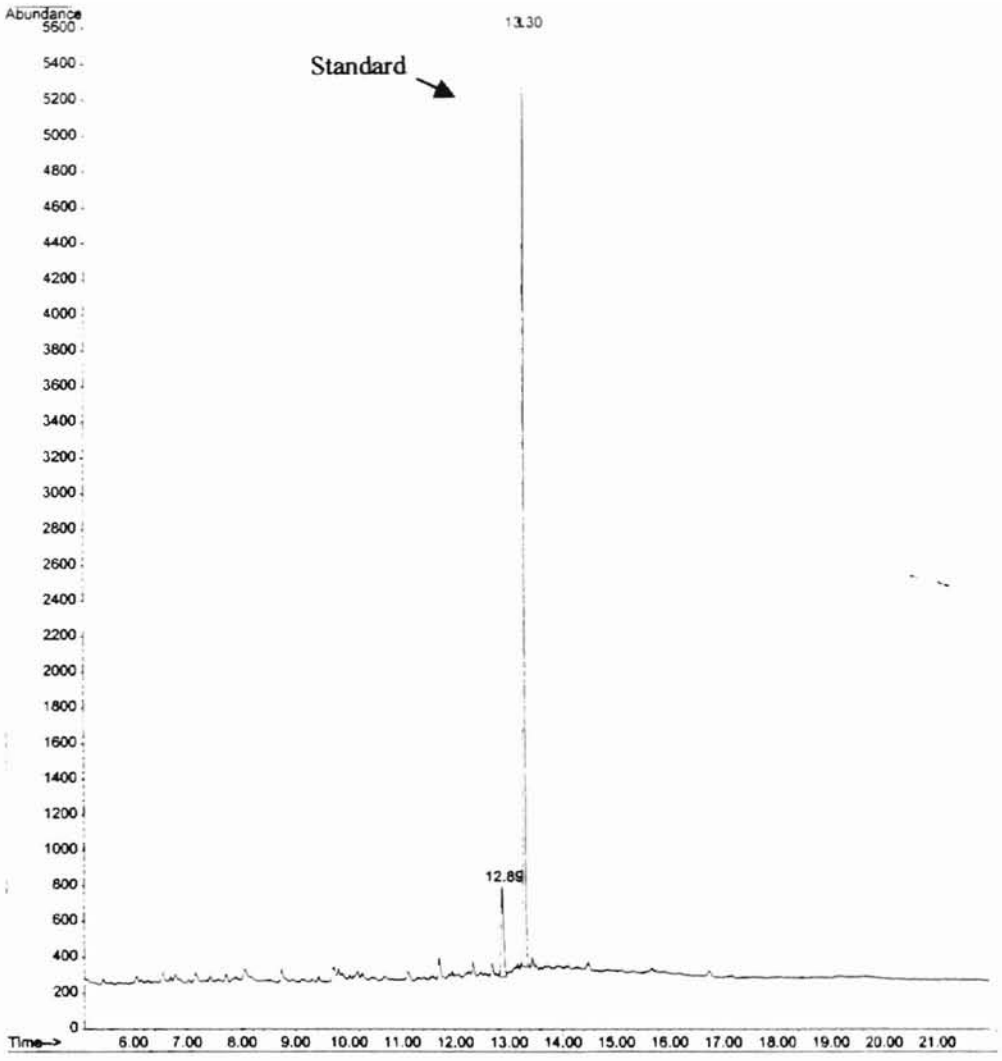
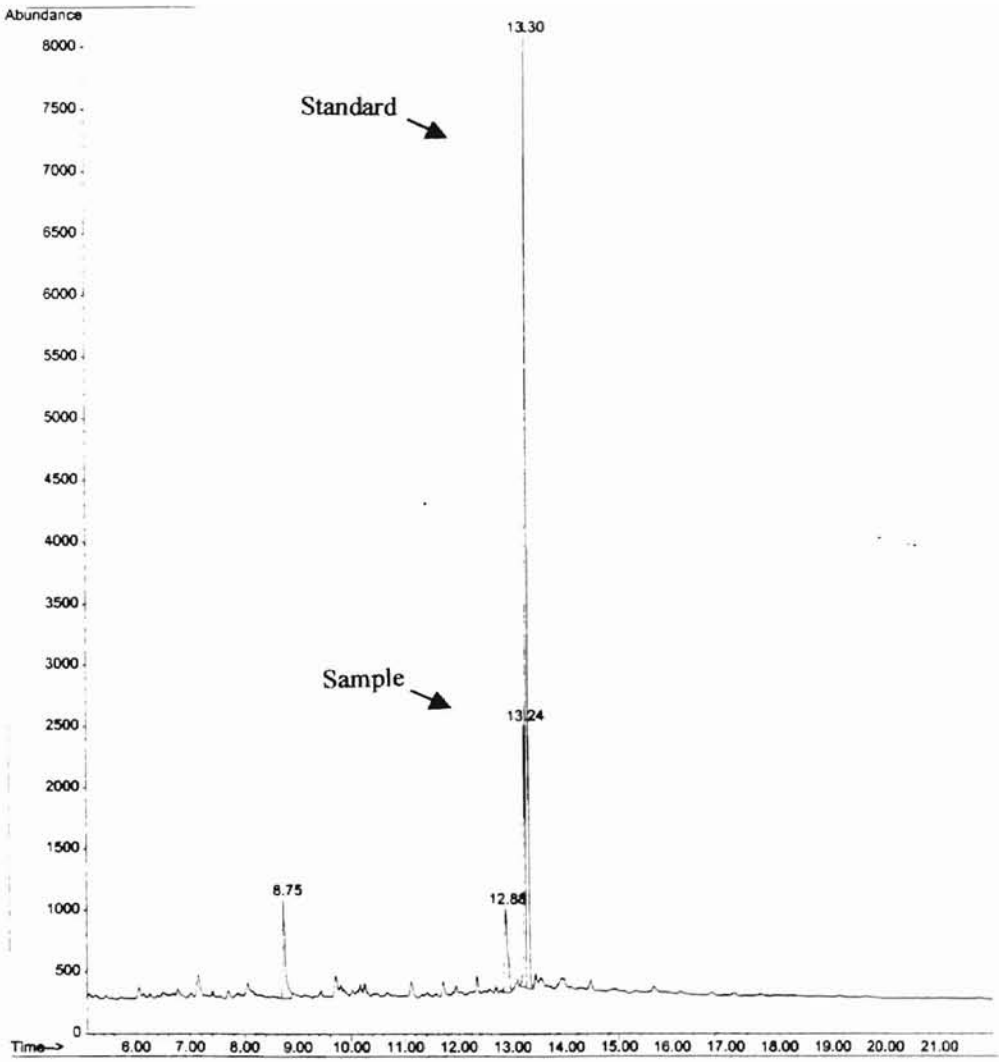


Fig.3.5 Fold increase in labeled and unlabeled PGE_2 in whole salivary glands produced as a function of exogenous unlabeled concentrations as measured by TLC, GC/MS and RIA. Data are experimental / control (w/o AA)

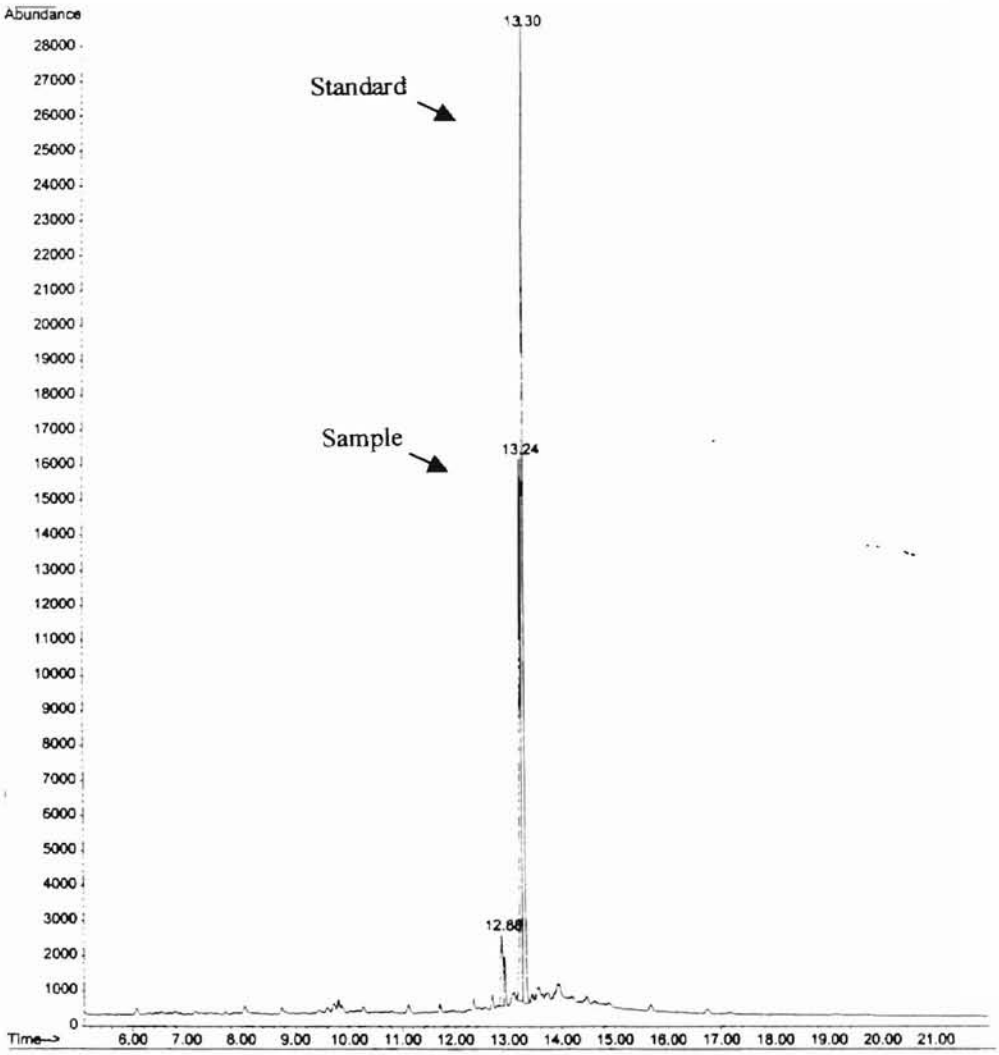


A)

Fig.3.6 GC/MS selective ion monitoring scan for PGE_2 levels using A) 0 μM B) 100 μM and C) 500 μM of exogenous AA respectively. The retention time of major PGE_2 peak in the column is 13.24 min.


Abundance
8000
7500
7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
Time-->
6.00
7.00
8.00
9.00
10.00
11.00
12.00
13.00
14.00
15.00
16.00
17.00
18.00
19.00
20.00
21.00
13.30
Standard
Sample
13.24
8.75
12.88

B)


Abundance
28000
27000
26000
25000
24000
23000
22000
21000
20000
19000
18000
17000
16000
15000
14000
13000
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Time-->
6.00
7.00
8.00
9.00
10.00
11.00
12.00
13.00
14.00
15.00
16.00
17.00
18.00
19.00
20.00
21.00
Standard
Sample
13.30
13.24
12.86

C)

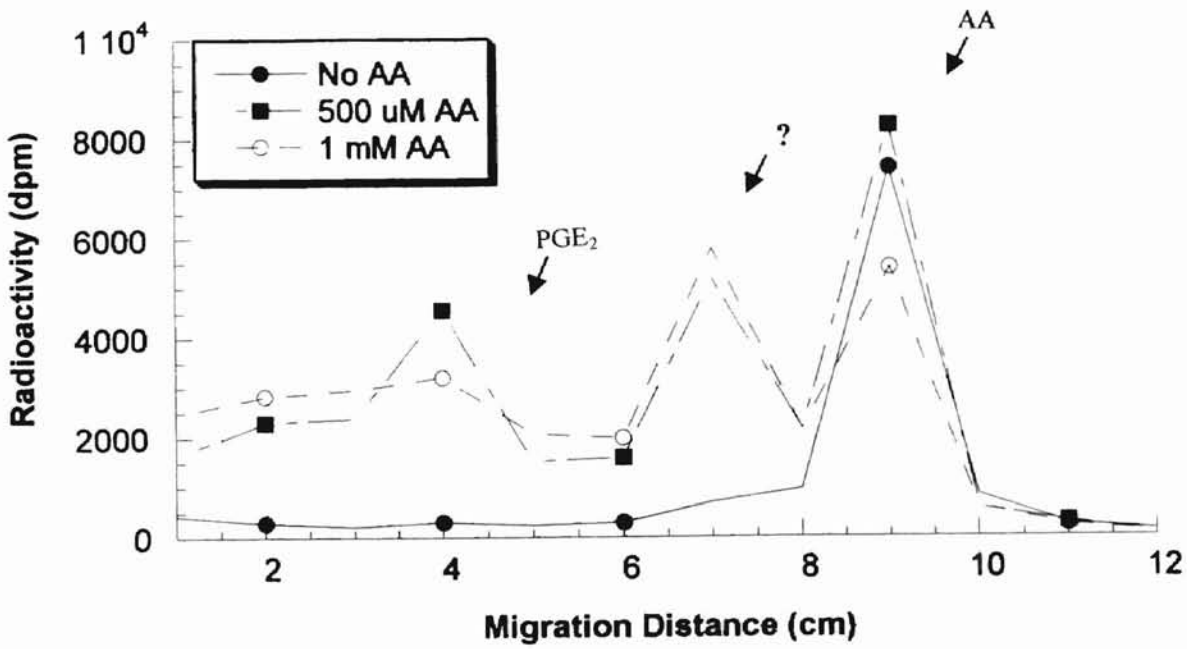


Fig.3.7 TLC Radiochromatogram of tick salivary microsomal fraction incubated with 0.1 μ Ci of [^{3}H]AA and 0, 500, 1000 μM unlabeled AA. All bands of sample lanes were cut and the radioactivity was measured by liquid scintillation counting. Arrows indicate migration distance of authentic PGE_2 and AA.

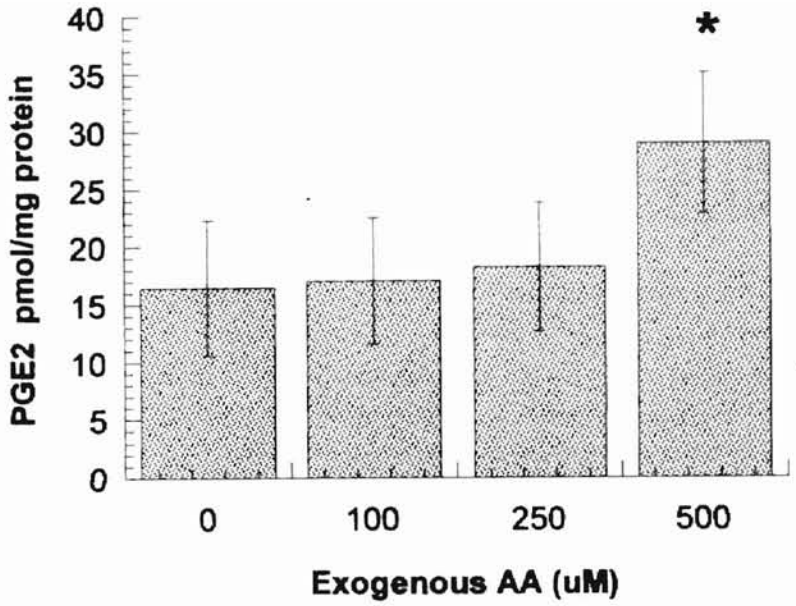
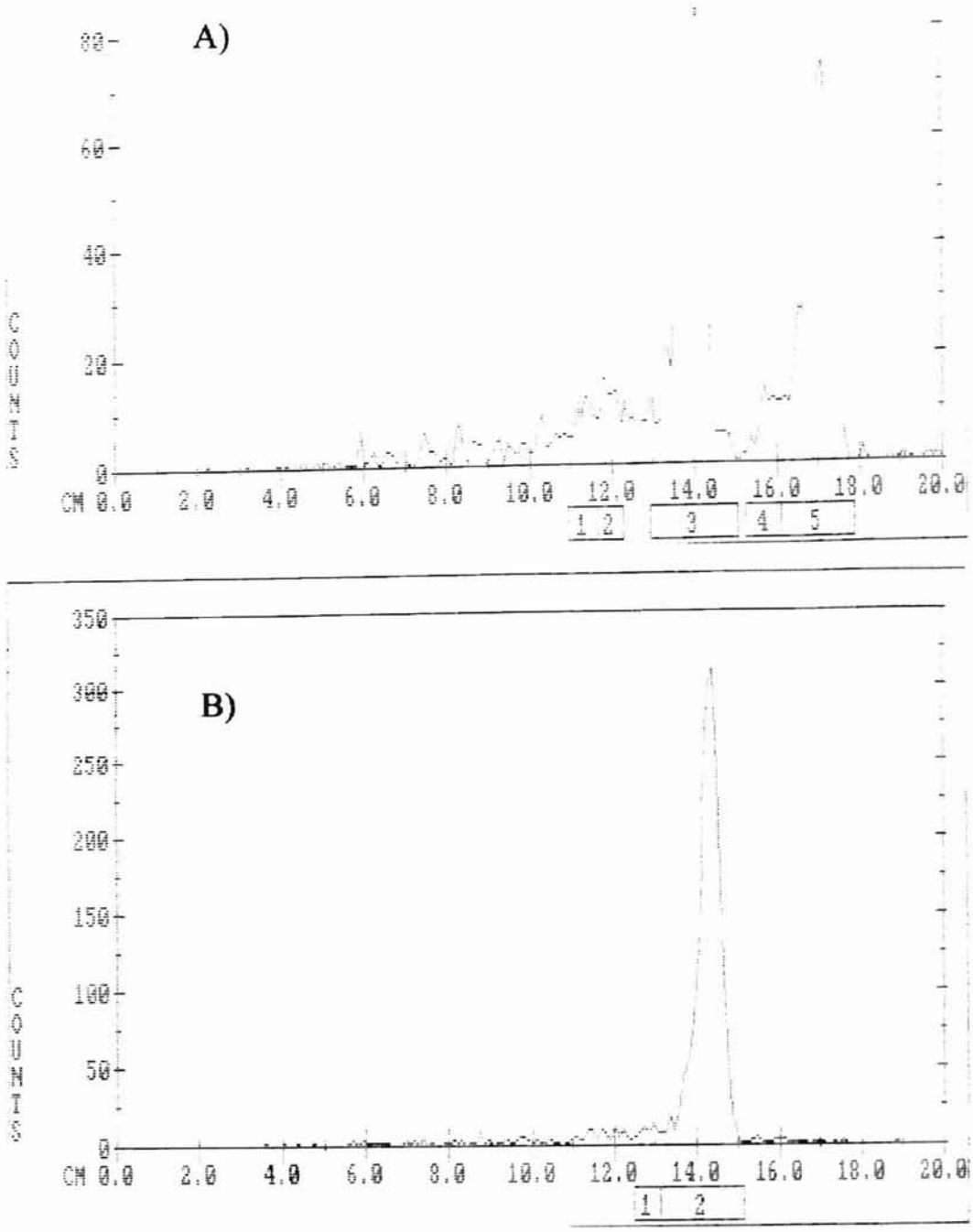


Fig.3.8 Increase in PGE_2 levels measured by RIA in the salivary gland microsomal fraction as a function of AA concentrations. Asterisk indicates significantly different from the control (w/o AA). Values are mean ± SEM, N = 8.

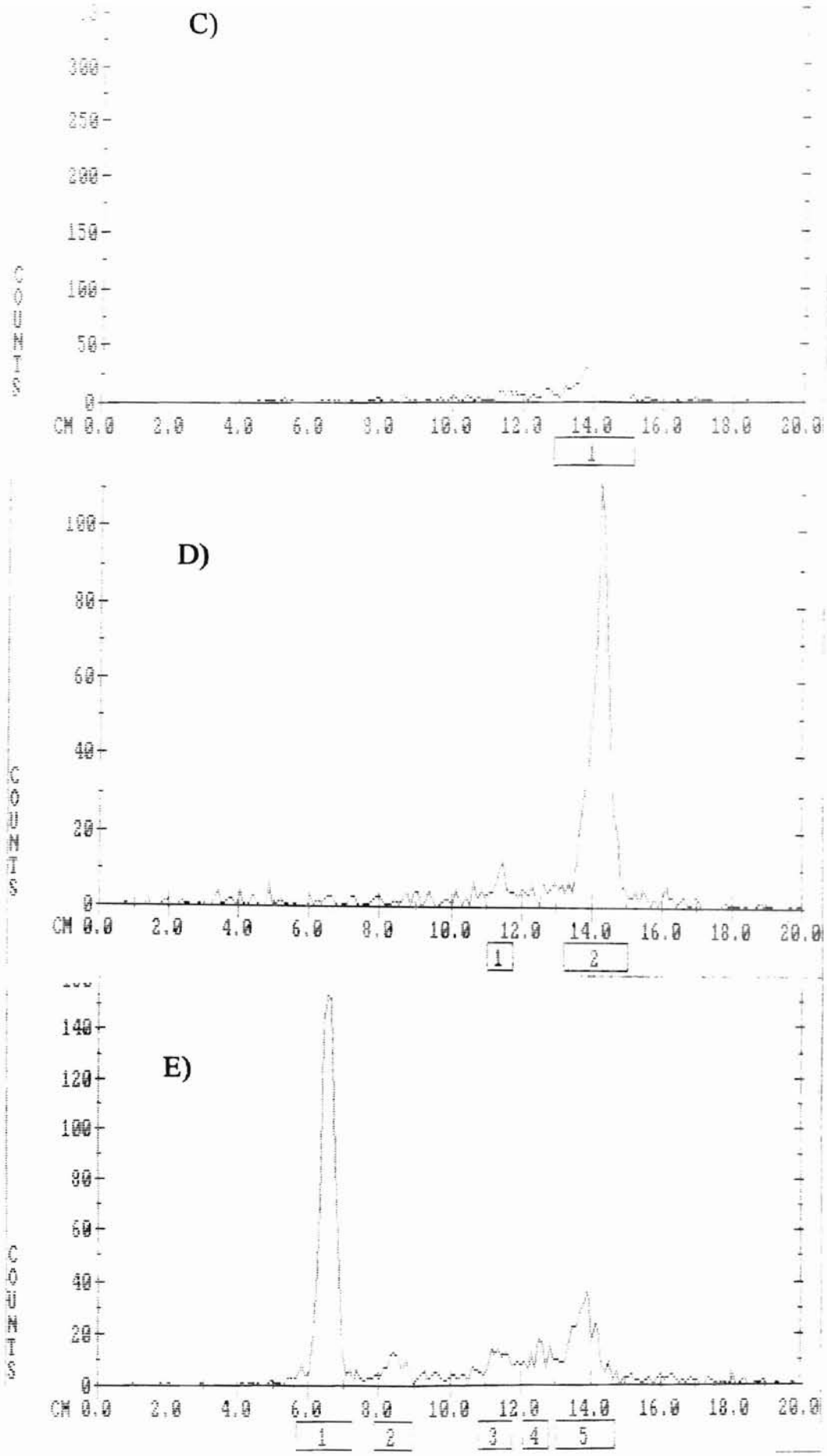
AA μM	Intact Cells (unwashed) ng/ Salivary Gland (sample 1)	Intact Cells (unwashed) ng/ Salivary Gland (sample 2)	Intact Cells (washed) ng/ Salivary Gland (n=1)	Microsomal Pellet pmol/mg protein (n=1)	Homogenate pmol/mg protein (sample 1)	Homogenate pmol/mg protein (sample 2)
0	4.18	1.31	1.14	9.58	101.74	191.96
250	5.73	4.54	1.19	19.93	136.57	172.9
500	13.95	24.68	5.07	25.81	332.22	209.23

Table 3.2 Levels of PGE_2 measured by GC/MS in intact cells unwashed and washed, microsomal pellet, and salivary gland homogenates in relation to different concentrations of exogenous AA. Washed cells were prepared by incubating them in 5 ml TC-199 MOPS for 1 hour at room temperature.



Migration distance (cm)

Fig.3.9 TLC Radioscan of A) intact salivary glands, B) intact salivary glands previously frozen, C) salivary gland homogenate, D) salivary glands microsomal fraction, and E) ram seminal vesicles. All were incubated with 0.1 μ Ci [^{3}H]AA for one hour at 37°C. PGE_2 standard appeared at 6-7 cm and AA at 13-14 cm, TG at 16-18. * Note difference in y-axis scale in each figure.


C)
D)
E)
COUNTS
CM 0.0 2.0 4.0 6.0 8.0 10.0 12.0 14.0 16.0 18.0 20.0

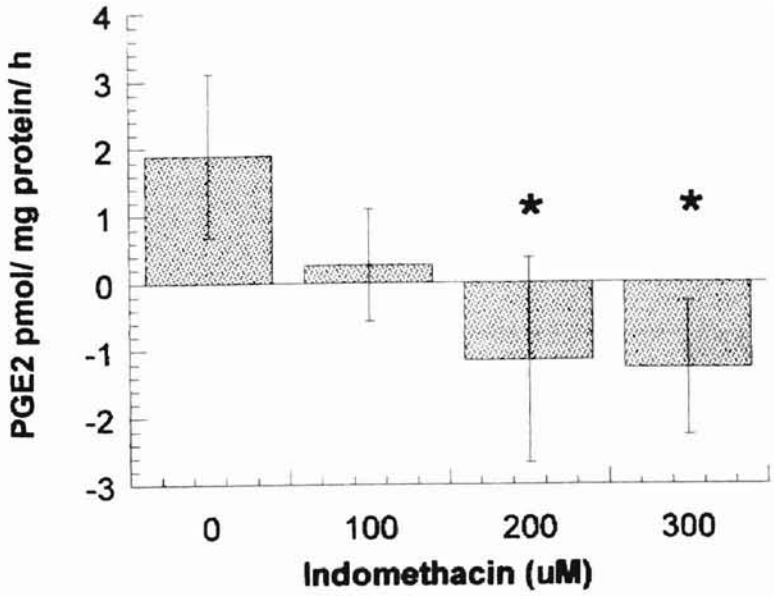


Fig.3.10 Inhibition of PGE_2 synthesis measured by RIA in the microsomal fraction of tick salivary glands. Samples were preincubated with varying concentration of indomethacin before 250 μM AA was added to all samples and incubated for 1 hour at 37°C. PGE_2 levels represent difference in the control (w/o exogenous AA) to that observed with exogenous AA ± the indicated concentrations of indomethacin. Asterisk significantly different from the control without indomethacin. Values mean ± SEM, N = 5.

	RIA pmol/ mg protein	GC/MS pmol/ mg protein
No AA	2.65	5.17
AA 250 μM	3.66	7.02
AA 500 μM	7.48	9.09
AA 500 μM + 100 μM Indo	6.15	4.06
AA 500 μM + 200 μM Indo	3.55	7.50

Table.3.3 Comparison of PGE_2 in the microsomal fraction measured by both RIA and GC/MS. The increase in PGE_2 correlated with AA concentrations. When indomethacin was added PGE_2 detection methods indicated a decrease in PGE_2 levels. Samples were preincubated for 30 min at 37°C with the inhibitor and then 1 hour with AA.

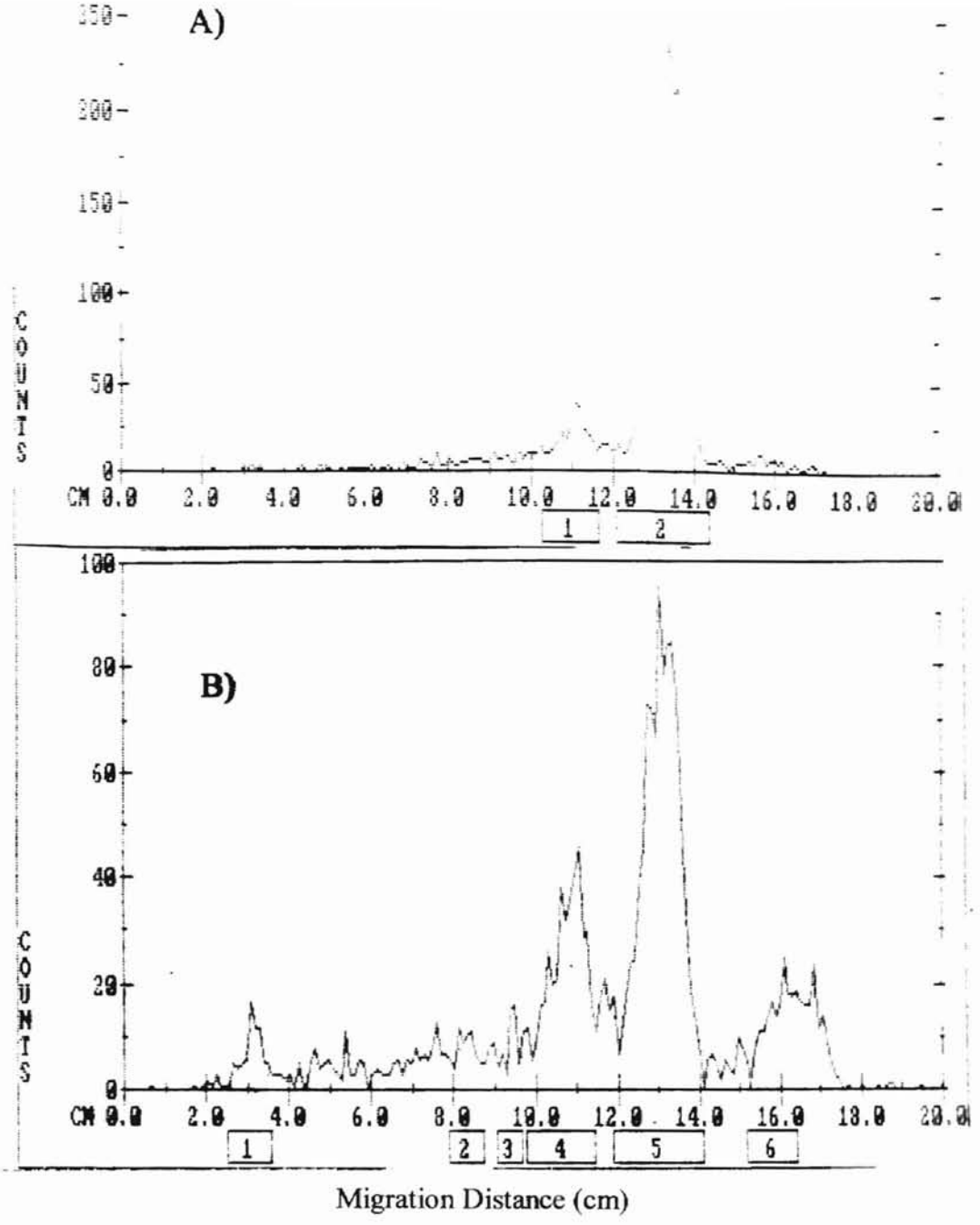
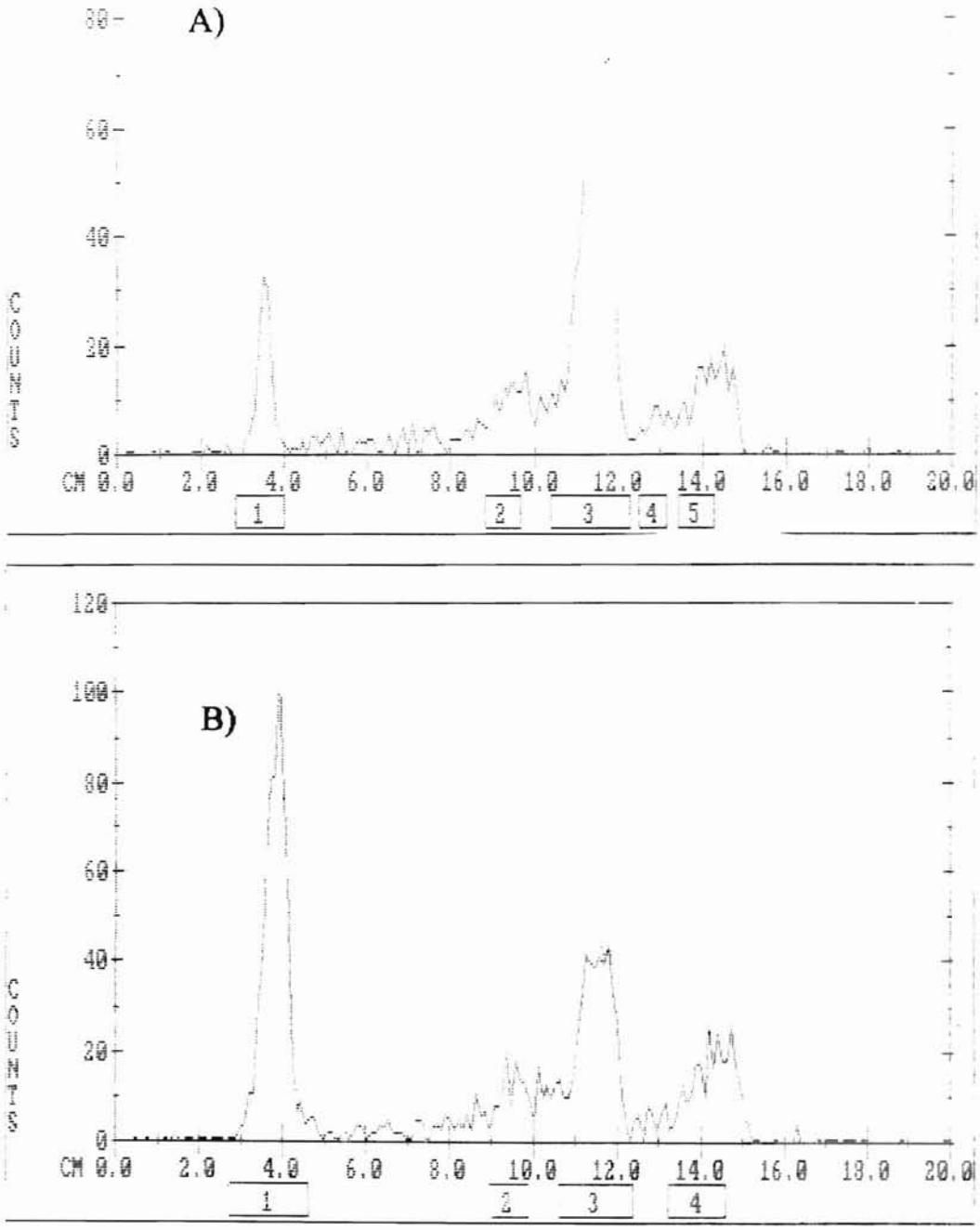
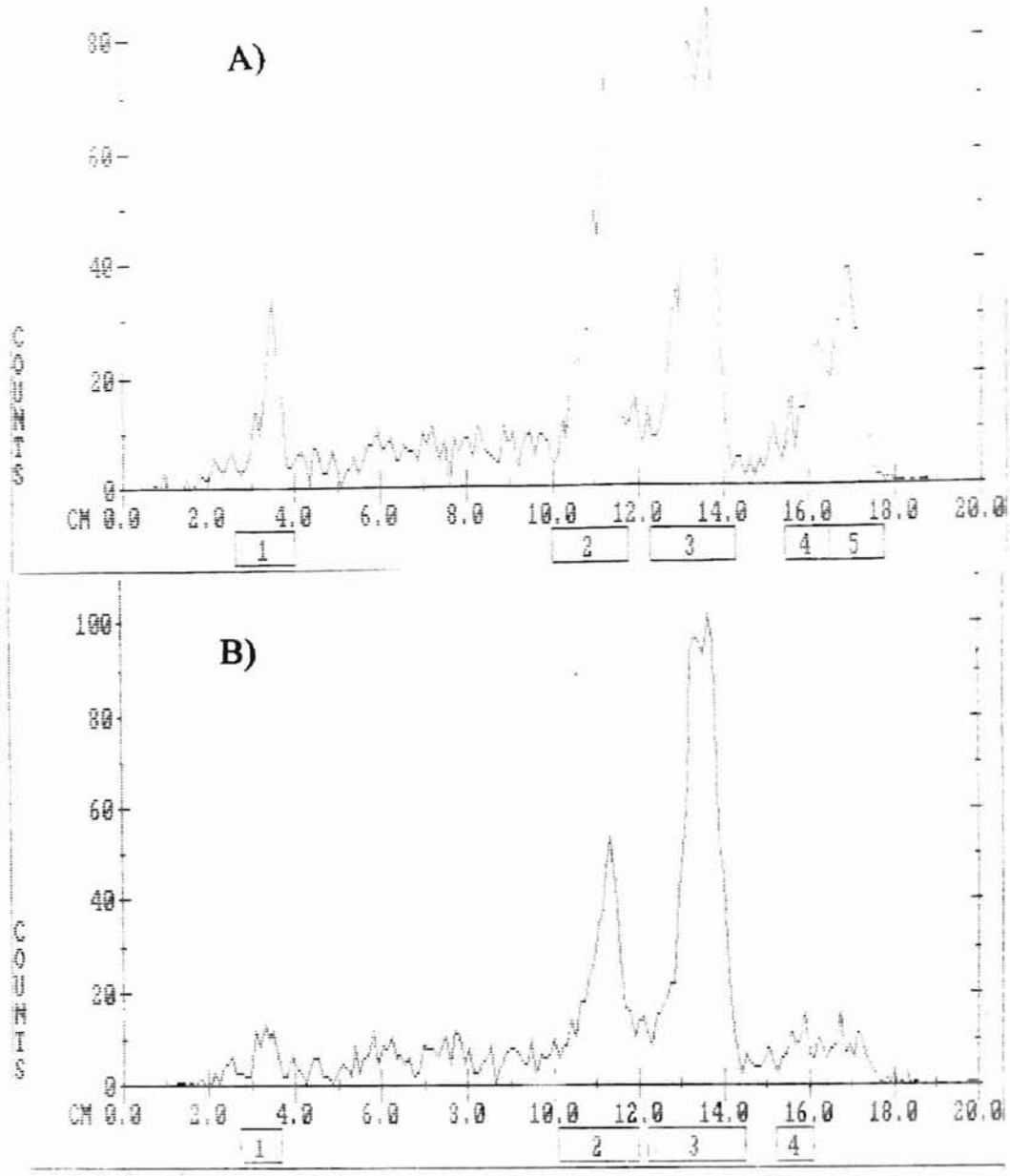


Fig.3.11 Time-dependency of AA metabolism in salivary glands. TLC radiogram of intact salivary glands incubated with 0.1 μCi for A) 1, and B)4 hours at 37°C. In A) peak 1 possibly represents a dehydration product of PGE_2. In B) peak 1,4,5,6, represent PL, a possible dehydration product or 5-HETE, AA, and TG, respectively. PGE_2 standard appeared at 7-8 cm.* Note the difference of y-axis scale in each figure.



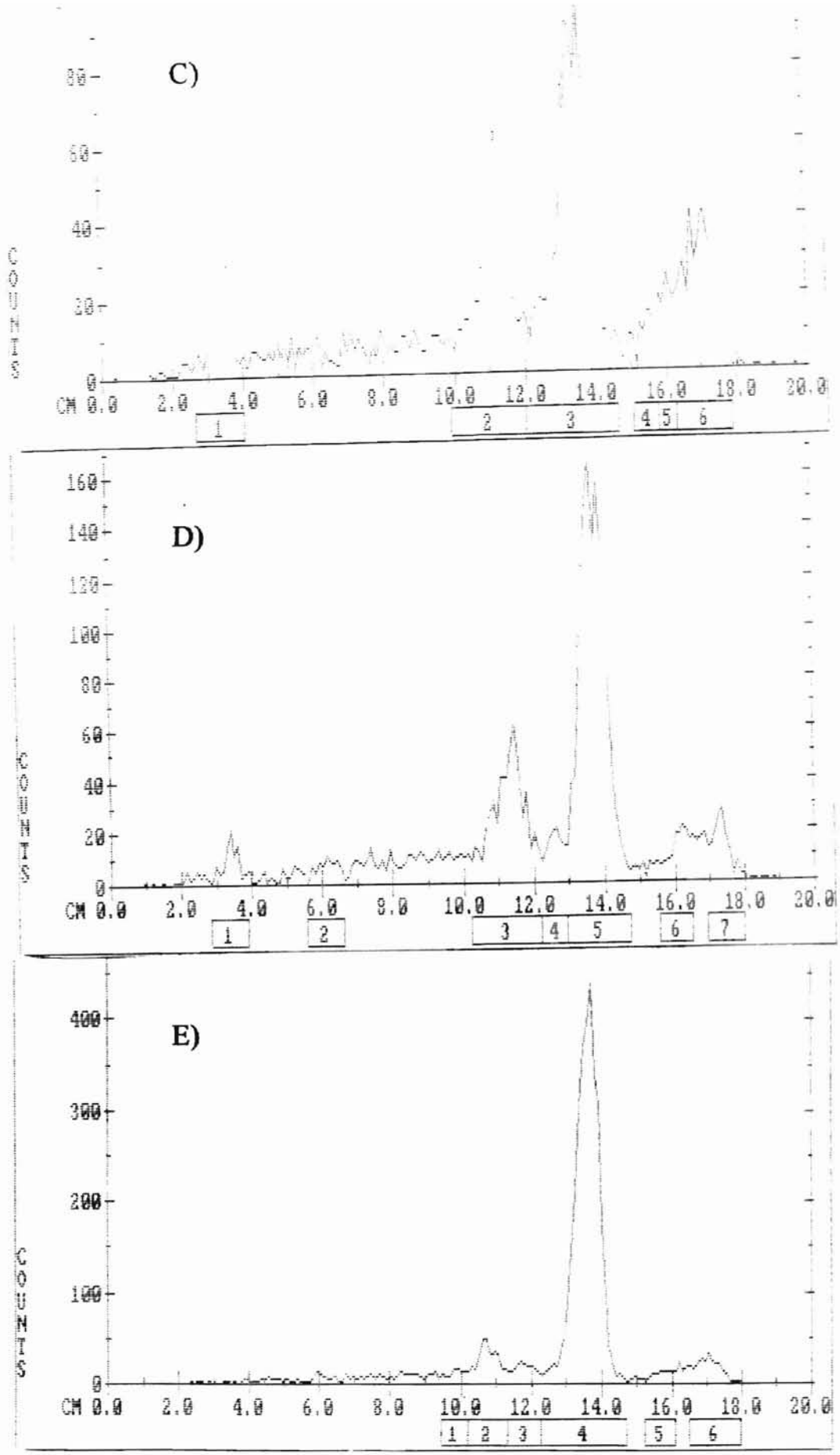
Migration Distance (cm)

Fig.3.12 Time-dependency of AA incorporation into membranous PLs of the salivary glands. TLC radioscan of intact salivary glands incubated with 0.1 μCi [^{3}H] for A) 1 and B) 4 hours, respectively. Peaks 1 and 3 in both a and b represent PL and AA respectively. * Note difference in y-axis scale in each figure.



Migration Distance (cm)

Fig.3.13 The effect of AA, dopamine, A23187 on AA metabolism in the salivary glands. TLC radioscan of intact salivary glands preincubated with 0.1 μCi [^{3}H]AA for 3 hours after which drugs were added and incubated for an additional hour. A) 0.1% ethanol, B) 500 μM AA, C) 10^{-4} dopamine, D) 10^{-4} A23187, and E) 500 μM AA was added with [^{3}H]AA and incubated for 4 hours. In a) peaks1, 2,3,4/5 represent PL, possible dehydration product of PGE_2, AA, and TG respectively. PGE_2 at 6-7 cm. * Note difference in y-axis scale in each figure.


C)
D)
E)
COUNTS
CM 0.0 2.0 4.0 6.0 8.0 10.0 12.0 14.0 16.0 18.0 20.0

	Sequences producing High-scoring Segment Pairs:		High Score	Smallest Sum Probability P(N)	N
1.	gi|2133552	peroxinectin - signal crayfish	175	8.2e-31	0
2.	gi|2707258	(AF035380) ovoperoxidase [Strongylocentro..	148	7.4e-24	0
3.	gi|4039144	(AF098717) peroxidase [Aedes aegypti]	133	1.5e-23	0
4.	gi|1945541	(AB003145) ovoperoxidase [Hemicentrotus p..	141	1.5e-19	0
5.	gi|2707260	(AF035381) ovoperoxidase [Lytechinus vari..	135	1.6e-18	0
6.	gi|400748	PEROXIDASE PRECURSOR	124	2.0e-18	0
7.	gi|4539761	(AF118391) salivary peroxidase [Anopheles..	93	1.5e-17	0
8.	gi|2429473	(AF025463) Contains similarity to Pfam do..	101	1.4e-15	0
9.	gi|3879398	(Z49129) similar to peroxidase; cDNA EST ..	99	2.7e-15	0
10.	gi|1397282	(U61948) similar to eosinophil peroxidase..	92	6.2e-15	0
11.	gi|630884	peroxidasin - fruit fly (Drosophila sp.)	81	2.8e-11	0
12.	gi|129823	LACTOPEROXIDASE PRECURSOR (LPO)	100	6.3e-11	0
13.	gi|3877355	(Z66520) similar to peroxidase; cDNA EST ..	72	1.6e-10	0
14.	gi|1504040	(D86983) similar to D.melanogaster peroxi..	73	1.7e-10	0
15.	gi|2239176	(Y11592) melanogenic peroxidase [Sepia of..	68	5.9e-10	0
16.	gi|3877971	(Z68005) Similarity to Drosophila peroxid..	73	1.6e-09	0
17.	gi|2117625	salivary peroxidase (EC 1.11.1.-) - human	89	2.2e-09	0
18.	gi|129826	MYELOPEROXIDASE PRECURSOR (MPO)	71	2.3e-09	0
19.	gi|129831	THYROID PEROXIDASE PRECURSOR (TPO)	65	3.0e-09	0
20.	gi|4587265	(AB022197) homologue of mammlian thyroid ..	72	1.2e-08	0
21.	gi|34719	(X15377) unnamed protein product [Homo sa..	67	1.5e-08	0
22.	gi|4557759	pre-promyeloperoxidase	67	1.5e-08	0
23.	gi|207435	(M31655) thyroid peroxidase [Rattus norve.	67	1.5e-08	0
24.	gi|88180	myeloperoxidase (EC 1.11.1.7), splice for.	67	2.0e-08	0
25.	gi|88182	myeloperoxidase (EC 1.11.1.7), splice for.	67	2.0e-08	0
26.	gi|4001752	(U68724) polysomal ribonuclease 1 [Xenopu.	69	2.7e-08	0
27.	gi|548478	THYROID PEROXIDASE PRECURSOR (TPO)	68	4.9e-08	0
28.	gi|88336	peroxidase (EC 1.11.1.7) precursor, eosin..	69	7.1e-08	0
29.	gi|4680721	(M17755) thyroid peroxidase [Homo sapiens]	65	7.3e-08	0
30.	gi|129830	THYROID PEROXIDASE PRECURSOR (TPO)	65	7.3e-08	0
31.	gi|339867	(J02969) thyroid peroxidase [Homo sapiens]	65	7.3e-08	0
32.	gi|4507655	thyroid peroxidase	65	7.3e-08	0
33.	gi|4503595	eosinophil peroxidase	69	7.5e-08	0
34.	gi|129832	THYROID PEROXIDASE PRECURSOR (TPO)	67	9.0e-08	0
35	gi|37251	(Y00406) precursor polypeptide [Homo sapi..	60	3.1e-07	0
36.	gi|5734613	(AB028841) BbTPO [Branchiostoma belcheri]	69	9.3e-07	0
37.	gi|543652	peroxidase-like protein (clone pL07) - sq..	67	1.1e-06	0
38.	gi|543651	peroxidase-like protein (clone pL04) - sq..	64	1.9e-06	0
39.	gi|4587263	(AB022196) homologue of mammlian thyroid ..	62	3.6e-06	0
40.	gi|129824	LACTOPEROXIDASE (LPO)	89	4.6e-06	0
41.	gi|1352739	EOSINOPHIL PEROXIDASE PRECURSOR (EPO)	69	7.1e-06	0
42.	gi|3875685	(Z81056) Similarity to eosinophil peroxid..	70	7.9e-06	0
43.	gi|2088832	(AF003385) Similar to peroxidase; R08F11...	63	1.1e-05	0
44.	gi|28898	(X17358) thyroid peroxidase (AA 1-876) [H..	52	1.2e-05	0
45.	gi|339871	(J02970) thyroid peroxidase [Homo sapiens]	52	1.2e-05	0
46.	gi|1777378	(L77979) eosinophil peroxidase [Mus muscu..	69	1.3e-05	0
47.	gi|2114321	(D88733) membrane glycoprotein [Equine he..	98	2.8e-05	0
48.	gi|494395	Canis familiaris	67	3.1e-05	0
49.	gi|2668615	(AF022977) similar to myleoperoxidase and..	71	3.4e-05	0
50.	gi|2606019	(AF030027) 71 [Equine herpesvirus 4]	96	8.2e-05	0

Table 3.4 Blast search results of 1.2 Kb segment amplified from tick salivary glands cDNA using peroxidase primers.

CHAPTER IV

DISCUSSION

Evidence is accumulating concerning the potential capability of tick salivary glands to synthesize prostaglandins. Tick salivary glands are capable of sequestering a considerable amount of AA from the host blood meal (Bowman *et al.*, 1995a), increasing about 40-fold, up to 1.64 µg / gland, in rapidly feeding ticks (Shipley *et al.*, 1993). Tick salivary glands also have a high amount of PGE_2, which increases from about 0.2 ng / unfed tick up to 80 ng / pair of salivary glands in ticks weighing 100-150 mg, then decreasing to about 20 ng / salivary gland pairs in rapidly feeding ticks (unpublished data). This high amount of PGE_2 in the salivary glands is puzzling relative to the known short half-life of prostaglandins in cells (Liu & Weller, 1990), and to the potency of prostaglandins, which are typically in picogram quantities in most tissues.

Previous attempts to show synthesis of PGs by isolated salivary glands have been either unsuccessful or disappointing (Bowman *et al.*, 1995; Pedibhotla *et al.*, 1995; 1997). The high content of both the substrate and the product might obscure the ability to detect the PGHS activity in these tissues. The exogenous AA used during *in vitro* PG biosynthesis studies might be diluted with the high content of the endogenous AA, though the existence of several pools that separate endogenous from exogenous AA cannot be excluded. Moreover, the stored PGE_2 in tick salivary glands might also dilute the newly synthesized product or have a negative feedback control on the *de novo* PG synthesis. Bowman *et al.* (1995b) has pointed to the capability of ticks *in vivo* to

synthesize PGE_2, which was secreted into the dopamine-induced saliva, though no *in vitro* synthesis could be detected. Pedibhotla *et al.* (1995; 1997) related that both the whole tick homogenate and the tick salivary glands microsomal-enriched fractions were capable of prostaglandin synthesis. Both studies showed the synthesis of small amounts of several prostaglandins; PGA_2/B_2, PGE_2, PGD_2, and $PGF_{2\alpha}$. However, the fractional conversion of AA to PG in tick salivary glands (0.35 pmol / mg / hour) was much lower than expected relative to the high molar concentration of PGE_2 both in the salivary glands and in the saliva (35 µM vs. 13 µM, respectively) (unpublished data). Moreover, we have recently shown, using GC/MS as a reference method, that no PGD_2 or PGB_2 can be detected in the salivary glands or saliva (unpublished data).

In the previous experiments, the concentration of exogenous AA used for *in vitro* PGHS activity detection was less than or equal to 10 µM. We tested a concentration range of 1 µM to 1 mM of exogenous AA. Using varying concentrations of AA we demonstrated a dose-dependent increase in PGE_2 levels in AA treated tissues above the control. The increase in PGE_2 levels was up to 50 ng / salivary gland using 1 mM AA. The levels of PGE_2 measured by RIA were confirmed by GC/MS.

The dose-dependency of PGE_2 production was also seen using labeled AA but was significant only when the labeled AA was spiked at a concentration of unlabeled AA above 10 µM. This might partially illustrate the difficulty in previous studies to detect PG biosynthesis activity using a low concentration of labeled AA that appear to be diluted by endogenous AA.

In addition to the high content of both the endogenous substrate and the product, several possibilities exist to explain the need of using higher than the standard concentrations of exogenous AA to detect PG biosynthesis in tick salivary glands. Gonchar *et al.* (1999) related that PGHS-1 could only utilize AA liberated from cellular membranes, which might be partially explained by the passage of AA to the cyclooxygenase active site through the hydrophobic channel, which is opened only to the membrane bilayer. The incorporation of AA into PL in cells needs several steps starting from the activation of the fatty acid via arachidonyl CoA synthetase followed by lysophosphatidyl acyl transferrase (LAT) (Balsinde & Dennis, 1996). In ticks, the uptake of AA seems to be an active process (Bowman *et al.*, 1995b). No calcium independent PLA_2 activity was reported in tick salivary glands. In mammalian cells this enzyme is responsible for providing lysophospholipid acceptors, the existence of which is considered the rate limiting factor for the incorporation of AA in resting cells (Daniele *et al.*, 1999). However, a calcium dependent PLA_2 activity was detected in both salivary glands and saliva in ticks (Bowman *et al.*, 1994; 1997). The intracellular PLA_2 might replace $iPLA_2$ activity in stimulated cells (Daniele *et al.*, 1999). The above data might indicate the need in ticks for a high amount of exogenous AA to saturate the lysophosphatidyl acceptors and accelerate the uptake of AA that forms the substrate for the membranous PGHS. Our results demonstrate that the incorporation of exogenous AA into PL was time dependent and took hours. Using our solvent system in TLC we cannot differentiate between PE and PC, though the salivary gland PLA_2 has no preference for either PL fraction (Bowman *et al.*, 1995d).

Another explanation for the high exogenous requirement is the possible existence of different pools of AA in the salivary glands. Tick salivary glands contain three types of acini (Fawcett *et al.*, 1986). Acini II and III undergo major changes upon feeding, paralleling a presumed need for prostaglandin biosynthesis during feeding. Many physiological and biochemical changes occur in the salivary glands during feeding including the increased expression of proteins (MacSwain *et al.*, 1982; Shelby *et al.*, 1987). The distribution of AA between TG and PL also changes during feeding. Shipley *et al.* (1993) reported that a major portion of AA in unfed female salivary glands was found in the TG fraction, which might benefit the tick as a source of energy. In contrast, the PL fraction was the major lipid containing AA during feeding, indicating a change in the distribution between PL and TG. Upon feeding, and instead of being a source of energy, AA might be a key substrate for the formation of biologically potent mediators to facilitate tick feeding. A better lipid fraction location for AA utilization may be phospholipids. The change in AA distribution might refer to the possible role of AA pools in the differential utilization of exogenous AA, which may also require higher concentrations of AA to maintain the suitable molar concentrations in the pools utilized by PLA_2 and PGHS. When the salivary glands were preincubated with the labeled AA, the unlabeled AA had an effect similar to that of the calcium ionophore in releasing the labeled AA from PL. This might indicate a possible role of high concentrations of exogenous AA in the remodeling of the endogenous pools. The PG synthase in tick salivary glands might have a low affinity for AA, thus with high Km and the requirement for high AA concentration to be activated.

Our results suggest that the microsomal fraction of the tick salivary glands have PG biosynthetic activity. We demonstrated PG synthesis using both labeled and unlabeled AA. The use of 500 μM AA resulted in a significant increase in the production of PGE_2 in both cases. Although AA concentrations less than 500 μM caused a significant increase in PGE_2 levels in intact cells, it is unclear why 500 μM was required for synthesis with the microsomal fraction. One explanation is that the incorporation system in the whole cells is more efficient than that in both the salivary gland homogenate and microsomal fraction. Bowman *et al.*, (1997) related a substantial reduction in the activity of the salivary gland PLA_2 after fractionating the salivary glands. This might lead to the requirement of higher concentrations of AA using the microsomal fraction by which a suitable amount of the substrate can get into contact with the PG synthase via the interaction with the endogenous AA remodeling process. We cannot exclude the possibility of co-factor requirements of the PGHS that might be different in the whole tick salivary gland than the standard cofactor mixture used in the microsomal fraction assay.

Our results indicate that the suicide inactivation of PGHS at high concentrations of AA (Hsuanyu & Dunford, 1992; Wu *et al.*, 1999) may not occur as readily in tick salivary glands.

Although the concentrations of PGE_2 measured by GC/MS were higher in the salivary homogenate than in the microsomes, the fold increase using 500 μM of AA was higher in the microsomes, which might reflect the existence of the PGH synthase enzyme in a more purified form (Table 3.2). An attempt to further purify the enzyme via solubilizing the microsomal fraction pellet using detergent was unsuccessful.

There was a major difference in the radioscan following incorporation of labeled AA between the intact cells, the homogenate, and the microsomal fraction (Fig.3.9). The conversion of the labeled substrate into products was higher in intact cells than in both homogenate and microsomal fraction. In addition, freezing the salivary glands seems to substantially reduce their capability of PG synthesis and AA incorporation into PL and TG. Pedibhotla *et al.*, 1997) demonstrated a decrease in PG biosynthesis activity in salivary glands frozen for more than 3 months.

Using unlabeled AA, the activity of prostaglandin synthesis in tick salivary glands was clearly higher than that reported by previous studies. In their work, (Pedibhotla *et al.*, 1995; 1997) measured the PGHS activity via the conversion of [^{3}H]AA into labeled prostaglandins, which was 0.35 pmol/mg protein/hour. Our results comparing PGE_2 levels between GC/MS and TLC indicate that the detection of PG synthesis in tick salivary glands using labeled AA alone might not be suitable. Two major reasons support this assumption. First, the high content of endogenous AA in tick salivary glands might dilute the labeled substrate, especially in our experiments where the specific activity of [^{3}H] AA is high (60-100 mCi / mmole). Secondly, the counting efficiency could affect the sensitivity of radioactivity detection. Using automated radioscanning, no major peaks were seen corresponding to PGE_2, although when we measured the radioactivity by scintillation counting we could demonstrate higher activity, which was comparable to those measured by both RIA and GC/MS (Fig.3.5).

We demonstrated that the increase in PGE_2 levels reaches up to 50 ng / intact salivary gland / hour at a substrate concentration of 500 μM. The PGE_2 synthesis in the microsomal fraction was less efficient and reaches approximately 1-2 ng / salivary gland

(30-60 pmol / mg protein). Using 10 μM AA, the mouse peritoneal macrophages show synthesis of approximately 140 ng PGE_2 / 10^6 intact cells and 55 ng PGE_2 / 10^6 cells in the microsomes (Gonchar *et al.*, 1999). Even though the apparent efficiency of prostaglandin synthesis in tick salivary glands may be lower than in the mammalian cells, our results using unlabeled AA clearly demonstrate a much higher rate than that reported in the previous studies.

The utilization of endogenous AA for synthesis was comparable to the exogenous AA though it took more time. The increase in PGE_2 levels was about 25 ng / salivary glands when intact cells were incubated for four hours. This might indicate that the tick salivary gland has a constitutive form of PLA_2 involved in releasing endogenous AA. In mammals, this isoform in resting cells is calcium independent ($iPLA_2$). This might imply the existence of an undiscovered calcium independent isoform of PLA_2 in the salivary glands, or the possible secretion of the Ca^{2+}-dependent secretory PLA_2 (Bowman *et al.*, 1997) that has an autocrine or paracrine role in releasing endogenous AA in the salivary glands of feeding ticks.

It is unknown at this stage what PGHS isoform exists in the tick salivary glands, or whether the tick has a unique PGHS. Several pieces of evidence may support the existence of an inducible form. First, the tick probably has no need for a constitutive activity prior to feeding. Upon feeding, the synthesis of PG is necessary to maintain the prolonged attachment. This might relate the tick enzyme to PGHS-2, which preferentially utilizes endogenous AA (Dubois *et al.*, 1998) and might be induced upon feeding by unknown inducers which the tick obtains from the host. Secondly, the utilization of exogenous AA represented by the fractional conversion was low and might refer to the

preferential utilization of endogenous AA, which is also a characteristic of an inducible enzyme (Dubois *et al.*, 1998).

Our results imply that the utilization of both endogenous and exogenous AA in the tick salivary gland microsomal fractions was inhibited by indomethacin, a potent and time-dependent inhibitor of both PGHS isoforms. The inhibition was seen only using high molar concentrations of indomethacin because of the competition between the inhibitor and both the exogenous and the endogenous substrate.

From the comparison of washed and unwashed salivary glands, the fold increase in unwashed cells was higher than that in washed cells. This may indicate that PGE_2 has positive rather than negative control on the *de novo* PGE_2 synthesis in tick salivary glands. Murakami *et al.*, (1997) concluded that PGE_2 might act as an amplifier of the delayed PG generation mediated by both $cPLA_2$ and PGHS-2.

In summary, our results strongly indicate that tick salivary glands are capable of substantial PGE_2 synthesis. This synthesis might be induced upon feeding by host factors and results in the increase in PGE_2 levels through feeding up to a tick weight of 150 mg. When the tick starts the fast feeding stage, PG synthesis might be shut off via unknown mechanism leading to the decrease in PGE_2 levels seen with engorged ticks. This, in turn, may signal the detachment of ticks from the host because of the need for higher amount of PG to maintain the feeding site. The incorporation and the remodeling of AA into salivary gland membranous lipid fraction, in addition to the high content of both AA and PGE_2, are all complicating factors in the study of PG synthesis in these tissues.

The PGE_2 synthesis was efficiently inhibited by indomethacin, a potent PGHS inhibitors, which might indicate the possible effectiveness of PGHS inhibitors in the pharmacological interference with tick feeding.

The PGHS enzyme in ticks might be different from the mammalian one especially in its possible requirement for a high amount of the substrate.

Using primers derived from conserved regions among mammalian PGHS isoforms, all cloned DNA segments showed no similarity to the mammalian enzyme. The only evidence for cloning a similar enzyme to a mammalian isoform in invertebrates came from coral with significant similarity in the protein sequence (Varvas et al., 1999).

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VITA

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Master of Science

Thesis: *IN VITRO* PROSTAGLANDIN E_2 SYNTHESIS IN THE SALIVARY GLANDS OF THE FEMALE LONE STAR TICK, *Amblyomma americanum* (L.)

Major Field: Biochemistry and Molecular Biology

Biographical:

Personal Data: Born in Homs, Syria, On November 8, 1971, the son of Nassouh Aljamali and Siham Alkasem.

Education: Graduated from Elzehrawee high school, Homs, Syria in June 1988; received Bachelor of Pharmacy and Pharmaceutical Chemistry from Faculty of Pharmacy, Damascus University in August 1993; received the Diploma and the Master of Clinical Biochemistry from Faculty of Pharmacy, Damascus University in October 1995 and March 1998, respectively.
Completed the requirements for the Master of Science degree with a major in Biochemistry and Molecular Biology at Oklahoma State University in July 2000.

Experience: Work for four years in the clinical lab of Al Assad University Hospital, 1993-1997, where I got the experience of techniques used in clinical blood analysis.

Scholarship: I was a grantee of the Fulbright scholarship during my graduate program for completing the degree of Master of Science at Oklahoma State University, 1998-2000.